Abstract

Collective cell migration is a key driver of tissue remodeling during development, wound healing, and cancer invasion. However, the mechanisms cells employ to move cohesively and the influence of the physical microenvironment on collective behavior have not been fully elucidated. Using two different engineered three-dimensional (3D) culture models, we show that cells require mechanical sensing to migrate collectively and that extrinsic physical forces in their microenvironment can influence the migratory phenotype. We conclude that physical forces and biomechanics play a vital role in collective migration, both in development and disease contexts.

To study the physical mechanisms of collective migration in mammary gland branching morphogenesis, we used 3D engineered tissues embedded in collagen, a fibrous extracellular matrix (ECM) protein found in the natural cellular microenvironment. We show directly and quantitatively that collective migration occurs via a dynamic pulling mechanism, with pericellular matrix alignment preceding translocation. Tensile forces increase at the invasive front of cohorts, serving a physical role and a regulatory one by conditioning the cells and matrix for further extension. These forces elicit mechanosensitive signaling within the leading edge and align the ECM, creating microtracks critical for migration. Cell movements are highly correlated and in phase with ECM deformations. Migrating cohorts use spatially localized, long-range forces and consequent matrix alignment to navigate the ECM.

We also determined how an extrinsic physical force in the microenvironment of solid tumors, elevated interstitial fluid pressure (IFP), influences collective cancer invasion. Elevated IFP is a characteristic feature of solid tumors; IFP rises steeply beyond the tumor periphery and plateaus at values as high as 50 mm Hg above a normal value of 0 mm Hg, resulting in outward fluid flow from the tumor core towards the periphery. We used a 3D engineered model of a human breast tumor to probe the effects of IFP on collective invasion. We found that IFP influences the motility and invasive behavior of cancer cells by regulating the expression of genes associated with migratory behavior (epithelial-mesenchymal transi-
tion (EMT) genes). The expression levels of these markers are both necessary and sufficient to drive invasive behavior in response to IFP.
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To my family.
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List of Abbreviations

3D  three-dimensional

2D  two-dimensional

αSMA  α-smooth muscle actin

bFGF  basic fibroblast growth factor

CDH1  E-cadherin

CRM  confocal reflection microscopy

ECM  extracellular matrix

EGF  epidermal growth factor

EMT  epithelial-mesenchymal transition

ERK  extracellular-signal regulated kinase

FAK  focal adhesion kinase

FGF  fibroblast growth factor

IFP  interstitial fluid pressure

IHC  immunohistochemistry

ILK  integrin linked kinase
KRT8  keratin-8
LPA  lysophosphatidic acid
MLC  myosin light chain
MLCK  myosin light chain kinase
MMP  matrix metalloproteinase
MRTF-A  myocardin-related transcription factor-A
PDGF  platelet-derived growth factor
PDMS  polydimethylsiloxane
PI3K  phosphoinositide 3-kinase
PKB  protein kinase B
ROCK  Rho-associated kinase
RTK  receptor tyrosine kinase
SNAI1  Snail
SRF  serum response factor
TFM  traction force microscopy
TGFβ  transforming growth factor-β
VIM  vimentin
List of Publications

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Chapter 1

Introduction

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1.1 Collective migration

Collective migration is a process by which cells move together cohesively as a group while 
maintaining connections to each other, as opposed to navigating their environment indi-
videntally [1, 2, 3]. Cells use this mode of migration in many biological contexts, including 
tissue morphogenesis, wound healing, and cancer invasion [4]. While single cell migration is 
important in several physiological processes such as the metastatic invasion of cancer cells 
and immune cell infiltration [5, 6], there are several advantages for cells to move collectively. 
For example, cohorts of cells can generate larger traction forces [7]. Moreover, collective
migration can achieve movement and structural changes at larger length scales, resulting in more complex tissue-level modifications within an organism.

Single-cell and collective migration share some common attributes, such as the involvement of mechanotransduction pathways (outlined below) and biochemical signaling, as well as interactions with the extracellular matrix (ECM). However, several characteristics distinguish collective migration. Unlike single cells, collectively migrating cells adhere to one another via cell-cell binding molecules that form adherens junctions, such as E-cadherins [8], which may be rapidly remodeled to allow for individual cell fluidity within a migrating cohort (Figure 1.1) [9, 10]. These junctional elements can link cytoskeletal proteins between individual cells, influencing network organization at larger length scales [11]. Integrins, in turn, enable contacts between the linked cytoskeletons and the ECM. Many collectively migrating cohorts also exhibit a distinct polarity in which “leader” cells guide “follower” cells in a particular direction [4, 12]. These cell types have different morphology and gene expression with “leader” cells being elongated and mesenchymal and “follower” cells being more tightly arranged and epithelial. Polarization can be achieved via external chemical and physical factors, such as soluble signaling molecules or ECM cues. In terms of movement through three-dimensional (3D) ECM, the migration of multiple cells is more constrained than that of single cells. Typical strategies of migrating cohorts are to push aside the ECM during migration, forming spaces large enough to accommodate multiple cells, or to remodel the ECM into directional tracks [13]. Collective migration can also occur along pre-existing basement membranes or vascular structures, which do not pose as much mechanical resistance [14]. In many ways, collective migration is quite distinct from single cell migration and more complex.

Cells can undergo collective migration in several different ways; they may move as clusters, multicellular strands, two-dimensional (2D) sheets, or ducts with a lumen. Moreover, migrating cohorts may consist of a single cell type or a combination of different cell types. The preferred migration mode depends on the biological context. For example, epidermal
keratinocytes move as a 2D monolayer during wound healing [15], border cells in *Drosophila melanogaster* embryos move as clusters [16], and branching mammary glands and angiogenic sprouts move as ducts with varying cell types and a luminal space within the epithelium [17, 18]. While it is understood that collective migration in every form requires cohesion between cells, polarization, and coordination of individual cell cytoskeletal activity, the underlying mechanisms behind collective migration modes remain elusive. Furthermore, the specific effects of environmental factors, such as physical and chemical cues, on collective behavior are also incompletely understood. Understanding the mechanisms that guide collective migration and the environmental factors that influence the process will provide insight to many biological processes such as those discussed below.

### 1.1.1 Collective migration in development and tissue regeneration

Collective migration is involved in several development and regeneration processes, such as oogenesis, branching morphogenesis, wound repair, and angiogenesis across different species. For example, during oogenesis in *Drosophila melanogaster*, border cell clusters migrate together in the egg chamber along germline nurse cells towards the oocyte [2, 19]. These cells
contribute to the formation of the egg shell, transport yolk proteins, and send patterning signals to the oocyte [16]. They migrate forward by sending out actin-rich protrusions at the leading edge while follower cells at the rear retract together via myosin-mediated coordination [20, 21]. Traction forces for migration are generated through E-cadherin cell-cell contacts with underlying nurse cells [22]. Collective migration is also observed during tracheal branching morphogenesis in the *Drosophila melanogaster* tracheal network. Here, an ectodermal germ layer thickening invaginates, exposing itself to a fibroblast growth factor (FGF) ligand [23]. Cells adjacent to the FGF sources take on a motile leader cell morphology, extending cytoskeletal protrusions and migrate towards the FGF source [24]. Less motile follower cells move together with the leader cells as a collective, forming a tracheal branch. This branching process then repeats to complete the tracheal network [25]. Mammary gland branching morphogenesis—in which luminal epithelial cells and myoepithelial cells undergo synchronous collective migration—is similarly dependent on growth factor signaling [17, 26]. However, in this case, there are no clear leader cells; cells forming a branch continuously move within the branch, exchanging positions with one another [27].

Angiogenesis, or the sprouting of blood vessels, is required both during development and tissue regeneration. Here, strands of endothelial cells penetrate tissues to form new vessel networks [28, 29]. Angiogenic sprouts contain a protrusive leader cell attached to a multicellular stalk via VE-cadherin [30]. Another form of collective migration for regeneration purposes is movement of keratinocytes as a 2D sheet to seal an epidermal wound. The single-cell layer later undergoes stratification to complete the formation of new epidermis [31]. The cells facing the open edge of the wound engage with collagen and fibrin via integrins to generate traction forces [32]. Cells further from the leading edge migrate along newly synthesized basement membrane [33]. The above examples demonstrate that collective migration plays a key role in many processes during normal development and responses to injury.
1.1.2 Collective migration in cancer

In many ways, cancer cells dysregulate normal developmental processes during epithelial tumor progression; this analogy also extends to collective migration. Unfortunately, the mechanisms and dynamics of collective migration in the context of cancer are difficult to study in vivo due in part to the long-term nature of disease progression. Nonetheless, cell culture models of collective cancer invasion have provided useful insights [4]. There are many similarities between collective invasion of cancer cells and collective migration during development and regeneration. For example, breast cancer invasions can be reminiscent of branching morphogenesis [34]. Moreover, morphogenic signals such as FGF are also involved in certain cancers [35]. Even ECM remodeling via matrix metalloproteinases (MMPs) has been shown to be crucial during the initial invasion of cancer cells in both 2D and 3D cell culture studies [13, 36]. One major difference between development and cancer, however, is that tissue organization is often lost in cancer invasion [4]. Moreover, unlike normal cells, cancer invasions are unresponsive to any mechanisms that would normally halt migration. Nonetheless, understanding the mechanisms driving collective migration in normal biological processes will likely also prove useful for understanding neoplastic progression.

Generally, a loss of epithelial markers and a gain of mesenchymal characteristics during the epithelial-mesenchymal transition (EMT) are thought to accompany malignant transformation [37]. However, there are many late-stage cancers that maintain epithelial characteristics while gaining some mesenchymal attributes, i.e. incomplete EMT [38]. The degree of cell-cell coupling, or completeness of EMT, dictates whether cancer cells move as individuals or collectively [39, 40]. Recently, it was found that complete EMT is not required for metastasis in lung and pancreatic cancers [41, 42]. Tumors can invade the surrounding stroma by collective invasion, and travel through blood and lymphatic vessels as clusters or multicellular strands to form distant metastases [4, 43, 44]. When cultured outside the body, many carcinoma explants primarily invade collectively [11, 45]. Immortalized epithelial cancer cell lines have also been observed to migrate collectively as 2D sheets when cultured in 2D or
3D strands in 3D ECM cultures [46]. Histological stains of epithelial cancer sections, such as breast tumors, have revealed that cells invading beyond the primary tumor boundary maintain cell-cell contacts [47, 48]. Elevated E-cadherin expression in tumors has even been correlated with poor prognosis and observed in metastatic lesions, suggesting that elevated levels of this marker may promote invasion [48, 49]. Furthermore, N- and E-cadherin have been observed during invasion in vitro and in vivo, in addition to other junctional proteins, such as tight junctions and gap junctions (which facilitate cell-cell communication), indicating that cohesion is beneficial for invasion [50, 51]. Clearly, collective invasion is an important mode of cancer cell migration and likely metastasis. A complete mechanistic understanding of collective cancer invasion and how this process is affected by the variety of physical and chemical cues in the tumor microenvironment may lead to treatment strategies designed to suppress invasion and metastasis.

1.2 Mechanotransduction in normal cells

Cells convert the physical signals they receive into biological responses via a process known as mechanotransduction [52]. In this way, mechanical forces dictate cell behavior. Mechanotransduction involves both the external environment and internal signaling [53]. The transmission of external forces to intracellular signaling is centered on proteins that are activated by force, such as integrins [54, 55] and T-cell receptors [56]. Many cellular phenotypes, including morphology, motility, and proliferation, are governed by external mechanical forces [57, 58, 59]. Thus, mechanotransduction is central to a variety of physiologically normal processes, including embryonic development, differentiation, wound healing, and angiogenesis [60, 61]. Defects in mechanotransduction are known to be involved in several diseases, including cancer [62].
1.2.1 Extracellular factors affecting mechanotransduction in normal cells

Most cells are anchorage dependent: they need to adhere to a substratum to prevent apoptosis and promote cell cycle progression [63]. Thus, the mechanical microenvironment is important for cell survival. Cells sense their environment via conformational changes in mechanically responsive proteins known as mechanosensors. Physical forces induce these conformational changes which result in downstream signaling inside the cell [60, 64]. Forces can originate from a variety of features, including the rigidity of the ECM, static or dynamic fluid, and tissue growth [52]. These forces are further broken down into specific types of loads that cells can detect. For example, forces incurred by blood flow include hydrodynamic pressure, shear stress, and cyclic strain; all of these help regulate endothelial cell behaviors [65], including cell reorientation [66].

Cells can also respond to mechanical loads by secreting biochemical factors, some of which result in subsequent ECM remodeling. Growth factors comprise one class of proteins that are important in this aspect. Transforming growth factor β (TGFβ) is sequestered in the ECM and is released when internal contractility of myofibroblasts is balanced externally by a stiff matrix, causing conformational changes in protein complexes embedded in the ECM. Free TGFβ starts a feed-forward loop, causing increased deposition of ECM proteins and additional (increased) expression of TGFβ [67]. Various other growth factors increase activity as a result of mechanical load, as evidenced by endothelial secretion of basic fibroblast growth factor (bFGF) in response to shear stress and hydrostatic pressure [68, 69]. Mechanical forces also regulate the expression of matrix remodeling proteins such matrix metalloproteinases (MMPs). This is observed in human monocytes/macrophages, which have been found to increase expression of MMPs under cyclic strain and thus contribute to ECM degradation [70].
1.2.2 Intracellular factors affecting mechanotransduction in normal cells

There are several intracellular components involved in receiving mechanical signals and eliciting a response (Figure 1.2). A feature that is particularly important to mechanical sensing is contractility; all cells have a network of cytoskeletal proteins (actin, microtubules, intermediate filaments) that aid in cell structure and mobility [63]. Cytoskeletal contractility creates a balance between intra- and extracellular forces acting on the cell and thus is important for cells to be able to respond to forces in the surrounding environment [71]. This balance exists so that mechanical forces in the microenvironment and internal cellular tension can work together to regulate cell behavior, evident, for example, in changes in fibroblast proliferation when matrix stiffness and actomyosin contractility are decoupled [72]. Moreover, external mechanical stimuli help define the state of the cytoskeletal components through various pathways. For example, it has been shown that tensile forces regulate the expression of α-smooth muscle actin (αSMA), a contractility gene, in osteoblasts [73] and that cytoskeletal tension in fibroblasts changes to match the stiffness of the substratum [74].

Communication between ECM and the cytoskeleton is mediated by mechanosensors, proteins or structures that can sense physical changes in the microenvironment and translate these into chemical signals inside the cell [61]. Mechanosensors are very diverse and exist everywhere in the body, from ears to intestine. For example, mechanoelectrical transduction channels in cochlear hair cells respond to sound vibrations to induce the signaling necessary in auditory sensation [75]. Another example is primary cilia in renal epithelia, which respond to fluid flow to maintain homeostasis [76]. The sensing mechanisms of many mechanosensors remain poorly understood.

The most well-studied mechanosensors are integrins, which contain extracellular, transmembrane, and cytoplasmic domains [77]. Integrins are composed of α- and β- subunits that form heterodimers [78]. Different types of integrins can bind to various ligands present in the ECM and induce signaling to regulate a variety of processes, including attachment,
migration, proliferation, and differentiation [79]. Through detection of external mechanical stresses, integrins promote changes in cytoskeletal structure and can activate signal transduction cascades [80, 81, 82]. Integrin activity is also essential for the formation of focal adhesions, which act as centers of mechanotransduction [83]. Focal adhesions are protein complexes localized at the plasma membrane that link the ECM to the actin cytoskeleton. In addition to integrins, focal adhesions include hundreds of proteins. The most well-characterized of these are talin, paxillin, vinculin, focal adhesion kinase (FAK), and Src family kinases, which act as signaling molecules [84]. The formation of focal adhesions is regulated by both external forces and cytoskeletal contractility [85].

Other intracellular components involved in mechanotransduction include G proteins, receptor tyrosine kinases (RTKs), extracellular-signal-regulated kinases (ERKs), and stretch-activated ion channels [52]. G proteins are localized at focal adhesion sites and can undergo conformational changes induced by mechanical stress to promote cell growth. G proteins
are activated in cardiac fibroblasts in response to stretch as well as in endothelial cells and osteocytes in response to shear stress [86, 87, 88]. RTKs are transmembrane proteins that dimerize to become activated, and are involved in integrin-mediated mechanotransduction downstream of G proteins. Dimerization is triggered by binding of the receptor to extracellular ligands such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), leading to further signaling [89]. RTKs can also activate ERKs which are important for gene expression and protein synthesis [90]. ERKs are kinases that play an important role in intracellular signaling, such as the activation of cytoplasmic and nuclear regulatory proteins. These kinases can be activated in response to mechanical stimuli. Shear stress and stretch have been shown to activate ERKs in aortic endothelial cells and pulmonary epithelial cells, respectively [91, 92]. Finally, stretch-activated ion channels allow ions such as Ca$^{2+}$ to move in and out of cells, which in turn regulate several cellular processes. Cell stretching has been shown to increase intracellular levels of Ca$^{2+}$ in several cell types [93, 94]. Intracellular Ca$^{2+}$ levels are also important for the activation of other proteins in the mechanotransduction signal cascade, such as ERKs [95].

1.3 Mechanotransduction in cancer cells

Over a decade ago, Hanahan and Weinberg defined several features of cancer that they considered essential for the acquisition of a malignant phenotype, including replicative immortality, evasion of growth suppressors, evasion of apoptosis, stimulation of angiogenesis, stimulation of proliferation, and invasion and metastasis [96]. Since then a flood of cancer research has led to modification and expansion of the proposed hallmarks; metastasis is one that persists [97]. Cancer is widely regarded as a disease of the cell, and cell behavior is directed by both biochemical and physical cues which can work independently or synergistically [98]. Accordingly, the tumor microenvironment has been shown to affect tumor progression [99, 100]. Changes in extracellular mechanical forces or defects in mechanosen-
sors can result in misregulation of signaling pathways inside the cell and ultimately lead to malignancy. Physical factors and mechanical forces that tumor cells encounter in the tumor microenvironment can in turn alter their behavior. Understanding how defects in mechanotransduction affect tumor progression will add to our fundamental knowledge of cancer biology and may suggest new approaches for treatment.

The invasion of primary tumors into their surrounding tissue and subsequent metastatic spread to other organs are among the largest obstacles to cancer treatment, and metastasis is the main cause of cancer-related deaths [101]. Metastasis relies on the ability of tumor cells to migrate from the primary tumor and form new lesions at distant locations [102]. Invasion and metastasis require physical interactions between malignant cells and their microenvironment—a process that inherently involves mechanosensing and mechanotransduction [62]. Both extracellular factors in the physical tumor microenvironment and intracellular factors within cancer cells contribute to mechanotransduction during invasion and metastasis. Importantly, identifying how mechanotransduction becomes abnormally regulated in cancer cells is necessary to understand the mechanisms that underlie invasion and metastasis.

1.3.1 Extracellular factors affecting mechanotransduction in tumors

The physical microenvironment within a solid tumor differs from that of normal tissue in several ways (Figure 1.3): uncontrolled proliferation of tumor cells results in increased mechanical compression in a spatially restricted environment [103]; increased production, alignment, and crosslinking of ECM components (of which collagen is the most prevalent structural protein) leads to increased ECM stiffness [104, 105]; poorly formed blood vessels and the absence of functional lymphatics lead to increased interstitial fluid pressure (IFP). These changes in the extracellular environment can alter the behavior of the tumor cells via mechanotransduction pathways which are important for both invasion and metastasis.
For example, mechanical compression can promote invasion and metastasis [106]. Compression has been shown to enhance cell-substratum adhesion in 2D cell culture compression assays [103]. Moreover, compression can facilitate invasion by increasing the release and activation of ECM-degrading MMPs [107]. Mechanical loading can also alter cell shape and motility through compression-dependent changes in cytoskeletal dynamics [108].

Figure 1.3: Cartoon illustrating the physical changes in the tumor microenvironment compared to that of normal tissue. (A) Normal tissue microenvironment. The microenvironment in normal tissues contains linearized blood vessels that perfuse the tissue. Lymphatic vessels are present to drain excess fluids and maintain fluid homeostasis. ECM proteins make up the loose connective framework. (B) Tumor microenvironment. Poorly formed blood vessels leak fluid and plasma macromolecules into the interstitium. Many solid tumors lack a functioning lymphatic system. There are larger amounts of ECM proteins that are highly aligned, crosslinked, bundled, and stiffened. In addition, uncontrolled proliferation of cells in a confined space results in mechanical compression.

The ECM is the framework for intercellular crosstalk, adhesion, and migration [109]. Solid tumors exhibit increased ECM stiffness and crosslinking, and changes in the structural components and mechanical properties of the ECM can promote an invasive phenotype in cancer cells [53, 62, 110]. For example, the mode by which tumor cells migrate is strongly dependent on the physical properties of the ECM [111]. Changes in ECM composition and architecture also affect the distribution and activation of soluble factors (e.g. growth factors,
cytokines, MMPs) that are themselves involved in cell behavioral changes and mechanotransduction [112]. ECM stiffness can promote the malignant behavior of tumor cells by increasing the expression and activity of adhesion receptors and activating mechanotransduction pathways [58]. For example, force has been shown to influence the development of focal adhesions since maturation of these complexes requires mechanical tension [113]. ECM stiffness also dictates cell behavior by increasing external resistance forces experienced by the cell [114]. Links to the ECM via integrins and focal adhesions can relay these stresses to the cytoskeleton, alter the balance of intracellular forces, and stimulate signal transduction cascades that influence cell behavior [53]. Moreover, increased ECM stiffness can disrupt epithelial polarity and induce migration and metastasis [115]. Cells have also been shown to migrate preferentially to regions of increased ECM stiffness via mechanotaxis/durotaxis [116, 117]. Finally, the crosslinking of ECM by lysyl oxidase, which can also stiffen the matrix by causing fibrosis, can promote tumorigenesis via enhanced integrin signaling [109].

ECM remodeling by tumor and stromal cells is important for both invasion and metastasis. For example, migrating tumor cells exhibit pericellular proteolytic degradation to make room for further migration [13]. Proteases such as MMPs are recruited to integrin assemblies and other adhesion receptors at the leading edge of a migrating cell to model and degrade the ECM [118]. Cancer cells have also been shown to realign their surrounding ECM perpendicular to the tumor boundary, altering its architecture for improved adhesion and migration and creating diverse routes for dissemination [119]. Migration is mediated by several types of proteolytic structures enriched with F-actin, β1-integrins, and MMPs which are key players in mechanotransduction [120]. Single cell migration can, however, also occur without proteolytic degradation under the mode of amoeboid migration [121]. The microscale architecture of the ECM, including the alignment of fibers and the location and size of pores, dictates the mechanisms of invasion and metastasis applied by cancer cells [122].

IFP and interstitial fluid flow have also been shown to affect the migratory and invasive behaviors of tumor cells. In a 3D culture model in which single tumor cells were suspended
in ECM, fluid flow was shown to increase the percentage of migratory cells as well as their speed [123]. In a similar study, interstitial fluid flow was shown to result in the upstream migration of cancer cells as a result of asymmetry in matrix adhesion stresses needed to balance drag from fluid flow [124]. The stresses induced by flow created a gradient of integrin activation across the cells. Components of focal adhesions, including FAK, paxillin, and vinculin, were shown be localized at the upstream side of the migrating cells.

1.3.2 Intracellular factors affecting mechanotransduction in tumors

It is well known that changes in mechanotransduction promote invasion and metastasis [125]. The intracellular factors affecting mechanotransduction pathways in tumor cells may be altered in response to changes in the tumor microenvironment or to genetic mutations and changes in gene expression within the tumor cells. Intracellular mechanotransduction can, in turn, lead to changes in gene expression to promote invasion and metastasis.

Cytoskeletal reorganization is important for changes in cell shape and motility, and, therefore, migration and metastasis [62]. Cytoskeletal tension is primarily regulated by ERKs and the Rho family of small GTPases. One effector of Rho is Rho-associated kinase (ROCK) which regulates actin cytoskeletal contractility via myosin light chain (MLC) phosphorylation [115]. Rho activity has been shown to be elevated in some tumors, though decreases in its activity have also been reported [126, 127]. Cytoskeletal tension is also affected by the mechanical properties of the ECM, such as stiffness and crosslinking [53]. Increased matrix stiffness promotes the clustering of integrins and the formation of focal adhesions while also increasing activation of FAK and ERK and enhancing ROCK-mediated cytoskeletal contractility [115]. ROCK is also involved in the disruption of adherens junctions and moving the tail end of the cell behind the leading edge to assist in cell locomotion [128, 129, 130]. Moreover, cell migration involves the extension of membrane protrusions resulting from the
cycling of actin polymerization and depolymerization which are regulated by Rho GTPases via the coflin pathway [131, 132].

ECM crosslinking has also been shown to result in the aggregation and clustering of integrins as well as enhanced signaling via phosphoinositide 3-kinase (PI3K) to induce invasion [109, 115]. Other components of focal adhesions have also been implicated in tumor progression. For example, Src activity has been shown to influence proliferation, invasion, and metastasis [133, 134]. Moreover, Src activation is required for ECM degradation during migration [135]. In 3D culture studies of breast tumor cells, Src activity was also shown to increase the strength of cellular forces on the ECM as well as the duration and length of cell membrane protrusions [136].

While some cells in the tumor become stiffer, metastatic cells are more deformable and exhibit reduced cytoskeletal stiffness [137]. Lower levels of integrin expression along with decreased adhesion to the ECM have been associated with oncogenic transformation [111, 138]. This increased deformability is correlated with enhanced metastatic potential. For example, enhanced deformability enables metastatic cells to move through tight spaces such as between endothelial cells during intravasation and extravasation [139].

In addition to regulating the cytoskeleton and associated proteins, mechanotransduction can lead to gene expression changes that promote invasion and metastasis. Cancer cells undergo a variety of genetic mutations and gene expression changes throughout tumor progression which can affect their interactions with the microenvironment and subsequent mechanotransduction. Mechanotransduction itself is one source of changes in gene expression in cancer cells. A major way that mechanotransduction can affect gene expression is via EMT. EMT, in which epithelial genes are downregulated and mesenchymal genes are upregulated, is thought to be an important mechanism in both invasion and metastasis [140, 141]. ECM stiffness has been shown to promote EMT, in which cancer cells acquire a migratory phenotype through a variety of pathways, some of which include key players in mechanotransduction, such as RTKs [142]. In one pathway, EMT results from stiffness-mediated
localization and signaling of Rac GTPases downstream of MMPs [143]. Mechanical stress and matrix rigidity can also induce EMT downstream of TGFβ [144, 145]. Furthermore, the activation of Rho proteins is thought to contribute to EMT via the loss of adherens junctions between cells and the gain of mesenchymal characteristics [146].

Induction of EMT in tumor cells, which affects cytoskeletal organization and cell-cell and cell-matrix adhesions, can also alter how the cells sense exogenous forces, and therefore their responses to those forces [147, 148]. The downregulation of keratins results in reduced cytoskeletal stiffness and greater cell deformability which directly influences the metastatic potential of tumor cells [149]. In addition to being more deformable than non-metastatic cells, metastatic cells also lose their anchorage dependence [150, 151]. Anoikis, or apoptosis induced by the loss of adhesion to the ECM, is suppressed in metastatic cells, allowing them to migrate and traverse through the bloodstream to distant organs [152, 153]. Anoikis is believed to be mediated by integrin signaling [154]. The activation of integrins and their associated proteins including FAK and integrin linked kinase (ILK) can suppress anoikis, indicating that mechanotransduction and apoptotic pathways are linked [155]. EMT can also suppress anoikis [156]. In particular, the downregulation of E-cadherin can protect cells against anoikis [157]. It is clear that several extracellular and intracellular components of mechanotransduction are altered in tumors, promoting progression to invasive disease. These outcomes suggest that mechanotransduction is another mechanism that can be hijacked to support malignant transformation.
Chapter 2

3D Cell Culture Models to Study Collective Migration

This chapter is adapted from the following publications:


2.1 3D epithelial tissues of defined geometry embedded in extracellular matrix

The architecture of branched organs such as the lungs, kidneys, and mammary glands arises through the developmental process of branching morphogenesis, which is regulated by a variety of soluble and physical signals in the microenvironment. Described here is a method created to study the process of branching morphogenesis by forming engineered 3D epithelial tissues of defined shape and size that are completely embedded within an ECM. The
detailed protocol is provided in Appendix A. This method enables the formation of arrays of identical tissues and enables the control of a variety of environmental factors, including tissue geometry, spacing, and ECM composition. This method can also be combined with widely used techniques such as traction force microscopy (TFM) to gain more information about the interactions between cells and their surrounding ECM (see Appendix B). The protocol can be used to investigate a variety of cell and tissue processes beyond branching morphogenesis such as cancer invasion.

2.1.1 Introduction

The development of branched epithelial tissues, known as branching morphogenesis, is regulated by cell-derived, physical, and environmental factors. In the mammary gland, branching morphogenesis is an iterative process through which guided collective cell migration creates a tree-like architecture. The first step is primary bud formation from the milk ducts, followed by branch initiation and elongation [158, 159]. Invasion of branches into the surrounding stroma is induced by the systemic release of steroid hormones at puberty. New primary buds then initiate from the ends of existing branches, and this process continues to create an epithelial tree [160]. Although many important biochemical signals have been identified, a comprehensive understanding of the cell biological mechanisms that guide this complex developmental process is currently lacking. Moreover, mechanistic studies on the influences of specific cues are difficult to deconstruct from experiments in vivo because precise spatiotemporal perturbations and measurements are often not possible.

3D culture techniques, such as whole organ culture, primary organoids, and cell culture models, are useful tools for systematically investigating the mechanisms underlying tissue morphogenesis [161, 162, 163]. These can be particularly useful for determining the influences of individual factors such as mechanical forces and biochemical signals on a variety of cell behaviors, including migration, proliferation, and differentiation [163]. Engineered
cell culture models in particular readily enable the perturbation of individual cells and their microenvironment.

One such culture model uses a microfabrication-based approach to engineer model mammary epithelial tissues with controlled 3D structure that consistently and reproducibly form branches that migrate collectively when induced with the appropriate growth factors. The major advantage of the model is the ability to precisely manipulate and measure the effects of physical and biochemical factors, such as patterns of mechanical stress, with high statistical confidence. This technique, together with computational modeling, has already been used to determine the relative contributions of physical and biochemical signals in the guidance of the normal development of mammary epithelial tissues and other branched epithelia [164, 165, 166, 167, 168]. Here we describe the methods for creating these model tissues, which can be readily extended to other types of cells and ECM gels, and which serves as a potential tool for the testing of therapeutics.

### 2.1.2 General schematic of epithelial tissue microfabrication

A general schematic of the microfabrication procedure outlining the experimental work flow is shown in Figure 2.1. An elastomeric polydimethylsiloxane (PDMS) mold is used to create cavities within an ECM gel that are then filled with cells. The end result is an array of epithelial tissues of identical geometry and spacing that are completely embedded within an ECM gel. A representative experiment uses EpH4 mouse mammary epithelial cells cultured in a gel of bovine type I collagen at a concentration of 4 mg/mL. To ensure the highest quality of engineered tissues, the techniques outlined in the protocol should be followed closely. Figure 2.2A and Figure 2.2B show low and high magnification views, respectively, of arrays of rectangular wells that have been molded into a type I collagen gel prior to cell seeding. The shape of the wells is determined by the shape of the features on the silicon master. Figure 2.2C shows rectangular wells in a type I collagen gel that have been filled with mammary epithelial cells, where excess cells have been washed off the surface of the
collagen. In this example, each 200 µm × 50 µm rectangular well contains approximately 80-100 cells.

2.1.3 Addition of growth factors induces collective migration

Roughly 24 hours after seeding, the tissues may be treated with growth factors such as HGF or EGF and cultured over several days to model collective migration during branching morphogenesis. Typically, collectively migrating cohorts begin to form as early as 4 hours after growth factor stimulation. Figure 2.3A shows representative results from rectangular mammary epithelial tissues within a type I collagen gel 24 hours after the microfabrication procedure after which cells have adhered to the collagen and to each other. No branches
Figure 2.2: Images taken during the microfabrication process. (A) Low and (B) high magnification phase-contrast images of rectangular cavities in type I collagen created using an elastomeric PDMS mold. (C) Cavities from (A) and (B) are filled with mammary epithelial cells. Scale bars, 100 µm.

are observed prior to growth factor addition. Figure 2.3B shows a representative rectangular tissue that has undergone branching 24 hours after the addition of HGF at 10 ng/mL. In this case, branches occur at the ends of the tissues (as opposed to the middle), where the cells experience the highest mechanical stress [165]. Multiple tissues of identical initial geometry in the same gel can then be imaged to determine population averages of branch location and branch length, enabling high-throughput analysis.

Figure 2.3: Microfabricated tissues undergo branching morphogenesis. (A) Phase-contrast image of a representative rectangular tissue 24 hr after microfabrication. (B) Phase-contrast image of a representative rectangular tissue that has started to undergo branching 24 hr after the addition of HGF at 10 ng/mL. White arrows indicate newly formed branches. Scale bars, 100 µm.
2.1.4 Immunofluorescence staining to visualize protein localization

Immunofluorescence staining of the tissue arrays in the culture model allows us to determine protein localization within a tissue with high statistical confidence. Figure 2.4A shows representative results from a branching rectangular mammary epithelial tissue stained for FAK. Creating frequency maps of tissues of identical geometry can be used to visualize the average spatial localization of proteins of interest within the tissues which can be compared to the localization of other proteins as well as branching activity. Figure 2.4B shows a frequency map of average FAK staining for 50 tissues showing FAK enrichment at the short ends of rectangular tissues where branching typically occurs.

Figure 2.4: Immunofluorescence staining of microfabricated tissues. (A) Immunofluorescence staining for FAK in a mammary epithelial tissue after branch initiation. (B) A frequency map of average FAK staining in 50 tissues. Scale bars, 100 µm.

2.1.5 Discussion

The protocol described above outlines a method to produce identical epithelial tissues of pre-defined shape, enabling spatial control of the mechanical stress experienced by cells in the tissue. An elastomeric mold is used to create cavities in type I collagen that are then filled with epithelial cells and covered with an additional collagen layer such that cells are completely encapsulated in a 3D collagen matrix environment. Further culture of these
tissues and treatment with growth factors to induce branching from the initial architecture make this system amenable for the study of branching morphogenesis of epithelial cells. There are several critical steps in the protocol. The first is lifting the PDMS molds straight upwards from the gelled collagen prior to cell seeding. Any horizontal movement during this step will distort the cavities, and they will no longer retain the desired geometry. Then, after the cavities have been seeded with cells, it is important to carefully wash off any excess cells that remain on the surface, as these may interfere with the behavior of the patterned tissues, particularly if they are very close to the tissues. Washing too rigorously, however, may result in cells being washed out of the cavities. Lastly, in the step when the cell-containing collagen gels are being immersed in culture medium, it is important to add the medium slowly and directly on top of the coverslip. Adding medium to the side of the dish away from the coverslip may result in detachment of the coverslip and the collagen lid. Each of these steps needs to be performed with care to achieve the best results.

Over the past few decades, several culture models have emerged for the study of epithelial morphogenesis; many of these involve the \textit{ex vivo} culture of intact organs or explants of organs. Major advantages of culturing tissue taken directly from an animal are that tissue architecture remains intact and that cell-cell interactions are maintained. However, these models are not readily amenable to studies investigating the specific effects of a particular cell type or physical microenvironment. Additionally, organs and organ explants can be fragile and difficult to culture \textit{ex vivo}. Others used primary mammary epithelial cells [169], in which gene expression can be more readily manipulated, though this method does not conserve \textit{in vivo} tissue architecture. Moreover, like intact organs, these cells can also be difficult to culture for long periods of time [162]. Therefore, there is a need for more robust culture models of epithelial morphogenesis that can be more precisely controlled and readily perturbed to gather reproducible data.

A widely used 3D cell culture technique is the formation of epithelial cell clusters embedded in collagen or other ECM proteins [168, 170]. Disadvantages of this technique, however,
include the inability to precisely control or predict the location of clusters within the gel, cluster size and shape, and branching sites. In addition, it is difficult to manipulate or measure the mechanical cues experienced by cells in different regions of the tissue clusters. Engineered tissues enable better control of these parameters.

The engineered tissue protocol described here is an easy to use, versatile, and reproducible 3D cell culture method that eliminates the heterogeneity commonly found in ex vivo culture systems and 3D clusters. Such engineered cell culture models provide a platform for more precise control of physical and biochemical signals, and results can be easily combined with computational models to predict the effects of perturbations or measure cellular forces using techniques such as TFM [165, 167]. A caveat of the model system, however, is that it does not completely replicate the cellular, chemical, and physical microenvironment within the mammary gland in vivo. Nonetheless, essentially all parts of the system are modifiable, including the cells used (in which gene expression can also be altered by transfection), tissue geometry, ECM composition, and other biochemical factors (e.g. growth factors). Primary mammary epithelial cells can also be cultured in this system; they form a lumen and undergo branching morphogenesis from the same locations as immortalized cell lines [164]. The model can also be modified to incorporate other cell types to more appropriately represent the native mammary gland microenvironment [171] or to investigate the underlying mechanisms that give rise to any branched organ.

2.2 3D culture model to study how fluid pressure and flow affect the behavior of aggregates of epithelial cells

Cells are surrounded by mechanical stimuli in their microenvironment. It is important to determine how cells respond to the mechanical information that surrounds them in order
to understand both development and disease progression as well as to be able to predict cell behavior in response to physical stimuli. Here we describe a protocol to determine the effects of interstitial fluid flow on the migratory behavior of an aggregate of epithelial cells in a 3D culture model. This protocol includes detailed methods for the fabrication of a 3D cell culture chamber with hydrostatic pressure control, the culture of epithelial cells as an aggregate in a collagen gel, and the analysis of collective cell behavior in response to pressure-induced flow. The detailed protocol is provided in Appendix C.

2.2.1 Introduction

In addition to the biochemical signals in the microenvironment, many aspects of the physical microenvironment can affect cell behavior [63, 172]. Physical factors including stiffness, pressure, flow, shear stress, and stretch can cause changes in cell behavior that help maintain tissue homeostasis during development; alterations in these physical factors are frequently associated with initiation and progression of disease [62, 173, 174]. For example, increased matrix stiffness is a telltale sign of cancer and can promote tumorigenesis [115]. A stiffer microenvironment can alter epithelial plasticity by promoting EMT in mammary epithelial cells [143], a process linked to cancer invasion. Furthermore, most malignant solid tumors have elevated IFP compared to normal tissue [175]. High IFP has been associated with poor prognosis and lymph node metastasis [176, 177].

Because of the connection between physical factors and disease, it is necessary to develop experimental models that recapitulate the various physical properties of the microenvironment [178]. Cells exist primarily in 3D within living tissues (though epithelial and endothelial cells can exist as quasi-2D monolayers). Interactions between cells, their neighbors, and the surrounding ECM are crucial for maintaining normal tissue function and homeostasis [179]. Therefore, 3D culture models are often used to recapitulate the structure and function of the tissue microenvironment [180, 181].
Solid tumors have higher IFP resulting from abnormal, leaky blood vessels and impaired lymphatic drainage [182, 183]. This feature has poor implications in cancer, as elevated IFP can lead to therapeutic resistance by hindering the delivery of drugs into solid tumors [177, 184, 185, 186]. IFP has also been shown to influence the migratory and invasive behaviors of single-cell suspensions of MDA-MB-231 breast cancer cells in collagen gels. In one study, a hydrostatic pressure differential of culture medium was established across the suspensions. Single-cell tracking showed that IFP increased the percentage of migratory cells and the speed at which they moved; cells were observed to migrate primarily in the direction of flow via autologous chemotaxis, a phenomenon that has been previously reported by the same group using modified 3D Boyden chambers [123, 187]. In similar studies, IFP was also found to affect the migratory behavior of MDA-MB-231 cells except that cells migrated against the flow direction particularly when seeded densely [124, 188]. The response of cancer cells to physical cues such as elevated IFP seems to depend on the context in which the signals are presented; neither of the previous models captures the behavior of an intact aggregate of tumor cells, suggesting the need for a new model. Here we describe a microfluidic approach to model the effects of IFP on an aggregate of tumor cells in 3D.

2.2.2 Construction of the microfluidic device

The basic structure of the 3D microfluidic device is shown in Figure 2.5. The PDMS framework is formed using a silicon master with features that produce channels that are approximately 20 mm long, 1 mm wide, and 1 mm tall. Holes are bored on either side of the channel; these will become media reservoirs. The PDMS chamber is then placed on top of a glass coverslip within a tissue culture dish with the open face of the channel against the glass. A needle-based technique is used to mold blind-ended channels in a neutralized collagen solution that gels within the PDMS chamber.
Figure 2.5: Schematic diagrams detailing preparation of PDMS chamber and channel surrounded by collagen. (A) Top and perspective views of a PDMS chamber for an individual channel with dimensions. (B) Top view of a chamber for an individual channel showing locations of holes A and B to be used as media reservoirs. (C) Top view of 100 mm tissue culture dish showing the placement of glass coverslips over a rectangular hole drilled in the middle of the dish. (D) Top view of a 100 mm petri dish containing acupuncture needles held in place by a stripe of PDMS. (E) Schematic detailing the preparation of an acupuncture needle with a PDMS support block. (F) Top view of a chamber conformally adhered to a glass surface with acupuncture needle setup prior to the addition of collagen into hole A. (G) Top view of a chamber containing a collagen channel with a cavity in the shape of the acupuncture needle from (F), with the channel adjacent to hole B filled with PDMS.

2.2.3 Cell seeding and set up of pressure profiles

Figure 2.6 outlines the seeding of cells in the 3D culture model as well as the set-up of a hydrostatic pressure gradient across the collagen channel. In this case, MDA-MB-231 breast cancer cells are seeded to form an aggregate, mimicking a solid tumor (Figure 2.6B), with the tip of the aggregate mimicking the tumor periphery and the base of the aggregate mimicking the tumor core. The cells can be cultured over several days. PDMS gaskets (Figure 2.6C) are used to alter the hydrostatic pressure differential across the cell aggregate (Figure 2.6D). Multiple gaskets can be stacked to achieve larger pressure differences. Pressure profiles can also be maintained over several days.
Figure 2.6: Schematic diagrams detailing the formation of 3D epithelial cell aggregates and the control of hydrostatic pressure profiles across the channels. (A) Side view of a blind-ended channel surrounded by collagen within a chamber being seeded with mammary epithelial cells via convection from a concentrated cell suspension in well B. (B) Phase-contrast image of a seeded cavity depicting the direction of convective flow during seeding. Scale bar 100 µm. (C) Perspective view of a PDMS gasket to be used to control the profile of hydrostatic pressure. (D) Perspective view of the culture model showing the creation of a hydrostatic pressure differential across the channel.

2.2.4 Immunofluorescence analysis

The cell aggregates can also be fixed at any point during culture by adding a fixative such as paraformaldehyde to one media reservoir (the higher pressure side) and allowing the fixative to flow through the cell aggregate-containing collagen gel by convection. Fixed samples can be stained using standard immunohistochemistry (IHC) techniques. Figure 2.7 outlines the procedure for removing the cell aggregate-containing collagen gels from the PDMS chamber and staining for a protein marker of interest. Figure 2.8 shows representative phase-contrast and confocal fluorescence images of MDA-MB-231 aggregates in our system under the control pressure condition \((P_{\text{base}}=P_{\text{tip}})\) stained for E-cadherin and cell nuclei.
2.2.5 Image analysis

In order to quantify collective invasion in our system, one can use image analysis software to measure the lengths of invasions as well as the density. Figure 2.9 provides an example of how invasion length is measured using a phase-contrast image. In this case, images were quantified using freely available ImageJ software.

2.2.6 Discussion

We have developed a culture model in which a defined hydrostatic pressure differential of culture medium may be applied across an aggregate of epithelial cells surrounded by a gel of type I collagen [189]. Our technique allows for the generation and control of the fluid pressure profile experienced by the aggregate of epithelial cells and enables us to examine the effects of
Figure 2.8: Visualization of cell aggregates using phase-contrast, small molecule dyes, and fluorescent antibodies. (A) An epithelial cell aggregate under a control pressure profile (no hydrostatic pressure differential across the channel) on Day 6 visualized using phase-contrast imaging. (B) Fluorescence image of the sample from (A) stained using the nuclear marker Hoechst 33342. Nuclei are shown in white. (C) Sample from (A) stained for E-cadherin (shown in green). Nuclei are shown in red. Scale bar, 100 μm.

IFP on collective migration of those cells. An important step in the protocol is insuring that the PDMS chamber is adhered to the glass coverslip, forming a tight seal. Similarly, PDMS gaskets should also be adhered to the PDMS chamber to prevent any leakage when setting up a pressure gradient. Additionally, when adding collagen to the channel, air bubbles should be avoided to prevent any disturbances in pressure-induced flow of culture medium through the gel. Each step in the protocol should be followed closely for best results.

An ideal model system is as close to in vivo conditions as possible; here, the 3D model mimics the physiological conditions of a dense tumor tissue. This approach allows for the direct visualization of cell migration and tumor invasion in 3D from a pre-existing aggregate.
Figure 2.9: Measurement of the invasion of cell aggregates. (A) Phase-contrast image of an epithelial cell aggregate on Day 6 under a pressure profile, obtained by holding well A at a higher hydrostatic pressure than well B. Invasion from the tip of the cell aggregate is shown with an arrow. (B) Fluorescence image of the sample from (A) stained using Hoechst 33342. Nuclei are shown in white. (C) Example of using the line tool in ImageJ to measure the length of the invasive protrusion from the epithelial cell aggregate tip in the sample from (A). (D) ImageJ measurement output. Scale bars, 100 µm.

as well as the analysis of changes in gene expression using in situ assays such as immunostaining and bulk analyses including Western blotting and real-time RT-PCR. The model can also be used as a platform to screen for therapeutics that inhibit cancer cell invasion under different pressure conditions. Although we use this model in the context of cancer, it could also be used to study the effects of pressure and/or fluid flow on other physiological or pathological processes.
Chapter 3

Dynamic Tensile Forces Drive Collective Cell Migration Through 3D ECM

This chapter is adapted from the following publication:


3.1 Introduction

Invasive collective migration, in which cells move coordinately through 3D ECM, is a key feature of morphogenesis and wound repair. The tubular structures of branched organs, including the *Drosophila* trachea and vertebrate vasculature, kidney, mammary, and salivary glands [17, 190], are generated by large-scale collective cell movements, tightly orchestrated spatially and temporally. These collective movements are also observed during the invasion
and metastatic spread of tumor cohorts [4, 46, 191]. While cell movement is thought to be initiated and driven by a variety of soluble cues [192, 193], collective migration is fundamentally a physical process wherein cells persistently penetrate a dense fibrillar matrix. During the migration of tumor cohorts through collagenous ECM, leader cells propel themselves forward by physically engaging with collagen fibers at the leading edge and proteolytically processing them at the cell posterior, leaving behind aligned ‘microtracks’ along which follower cells can migrate [13]. A similar mechanism for collective migration has been observed in 3D cocultures of carcinoma cells and fibroblasts, where fibroblasts act as leader cells and create tracks along which cancer cells follow [194].

The adhesive interactions with the ECM and the indispensable role of integrins [11] and Rho signaling observed in these and other studies in both 2D and 3D systems [7, 195, 196, 197] strongly suggest that collective migration in these cases requires mechanical force. Mechanical forces that arise during collective migration of cellular sheets along flat surfaces and their spatiotemporal variations have been characterized extensively [7, 197]. However, although they are highly informative about collective behaviors, these models do not fully replicate the mechanical, structural, and geometrical features of inherently 3D collective migration processes, including angiogenesis, branching morphogenesis, and most cases of cancer invasion. In particular, cellular sheets crawling on surfaces grip the underlying substratum tangentially to propel themselves forward, unconstrained by frontal physical obstacles. In contrast, a 3D matrix provides physical support to an invading cellular collective but also impedes movement by providing frontal constraint. Furthermore, whereas the mechanically defined materials used in 2D studies enable full quantification of cellular tractions, they do not faithfully mimic the complexity of physiological matrices that respond to these very tractions by altering their structural and mechanical properties [165, 198, 199], likely influencing the migration process. We thus set out to characterize the forces and ECM deformations arising during collective migration through physiological 3D matrices, which have not been fully elucidated.
3.2 Results

3.2.1 Epithelial tissues migrate collectively by pulling on the ECM

We used arrays of microfabricated tissues to investigate the physical mechanisms that drive invasive collective migration. This approach generates hundreds of regularly spaced 3D epithelial tissues of defined size and shape embedded in a matrix of native type I collagen [164]. In this system, cells invade collectively from predictable and reproducible locations within the tissues (Figure 3.1A), enabling high-throughput analysis with high statistical confidence [164, 165]. Importantly, unlike classic in vivo models, these platforms enable the control, measurement, and manipulation of mechanical parameters.

To measure matrix deformations and the corresponding mechanical forces that accompany collective cell migration, we monitored the motion of fluorescent beads embedded within the collagen surrounding the tissues. As cells invaded collectively into the collagen, the beads were displaced incrementally toward the extending cohort (Figure 3.1B), suggesting that the invading cohort generated a tensile force which pulled on the surrounding matrix. To characterize these forces, we reconstructed the surface of the tissue (Figure 3.1C, D), quantified bead displacements, and estimated the associated strains and tractions (i.e. forces exerted by the cells). It should be noted that the goal of the quantification was to assess the spatial distribution of the forces and their directionality rather than to provide an absolute measure of magnitude. Absolute quantification in this case is challenging, owing to the spatial and temporal variations in matrix mechanics [200, 201] as well as cell-induced heterogeneities and anisotropies (i.e. collagen alignment, discussed below). Bead motion was directed inward toward the collectively migrating cohort. Tractions were localized to the invasive front (Figure 3.1E) and, importantly, were tensile in nature, indicating that the cohort translocated forward by pulling on the matrix.

To determine the generality of this physical mechanism, we measured matrix displacements in classic models of mammary epithelial branching morphogenesis, itself a form of
collective migration [27, 202]. Branch initiation and extension from clusters of mammary epithelial cells (Figure 3.1F, G) and primary mammary organoids (Figure 3.1H, I) were accompanied by inward-directed displacements, which again localized to narrow (∼50 µm wide) regions ahead of each branch and propagated up to 150 µm away. Collective invasion of cancer cells also proceeded via a pulling mechanism (Figure 3.1J, K). However, displacements in this case were considerably smaller and more diffuse than those arising during collective migration of non-neoplastic epithelial cells.
Figure 3.1: Epithelial cells migrate collectively by exerting tensile forces on the surrounding 3D matrix. (A) Confocal fluorescence images showing collective migration of mammary epithelial tissues labeled with LifeAct-GFP (actin, green) and H2B-mCherry (nuclei, red) in type I collagen gels over 24 hours. (B) Confocal slice of tissues at 0 and 20 hours with bead displacements superimposed. (C) Confocal stacks of a representative tissue were used to reconstruct (D) the surface of the tissue and the migrating cohorts. (E) A map of estimated traction forces exerted by collectively migrating epithelial cells. (F) Phase-contrast image of a cluster of mammary epithelial cells in a type I collagen gel undergoing collective migration.
(G) Confocal slice of the mammary epithelial cell cluster in (F) labeled with LifeAct-GFP with bead displacements superimposed. (H) Phase-contrast image of a primary mammary organoid in a type I collagen gel undergoing collective migration. (I) Confocal slice of the primary mammary organoid in (H) labeled with LifeAct-GFP with bead displacements superimposed. (J) Phase-contrast image of a cluster of breast cancer cells in a type I collagen gel undergoing collective invasion. (K) Confocal slice of the cluster of invasive cancer cells in (J) labeled with LifeAct-GFP with bead displacements superimposed. Scale bars, 50 µm.

3.2.2 Dynamically migrating cohort movements correlate with ECM deformations

Live imaging revealed the dynamics of collective migration and the interactions between the cells and their surrounding ECM (Figure 3.2A). Collectively migrating cohorts exhibited dynamic changes in shape during migration (Figure 3.2B, C). The projected area of the cohort increased relatively linearly in time while its length fluctuated (Figure 3.2D). Comparing bead displacements to changes in cohort length revealed that beads adjacent to the extending collective moved coordinately and in phase with the cohort (Figure 3.2E-G). Beads far from the migrating cohort (>50 µm) showed little displacement, and their movements did not correlate with that of the cohort (Figure 3.2G). We also noted that cohorts continuously exert a tensile force on the ECM during extension, holding the ECM taut during migration.

3.2.3 Cytoskeletal tension drives collective migration

To determine whether active contractility was required for collective migration, we blocked cytoskeletal tension by treating the invading tissues with blebbistatin, which inhibits myosin ATPase (Figure 3.3A). Disrupting cell-generated forces significantly impaired the extent of collective migration (Figure 3.3B). Similar results were observed when treating tissues with Y27632, a Rho kinase inhibitor (Figure 3.4A, B). Conversely, enhancing cellular contractility by treating with lysophosphatidic acid (LPA), an activator of Rho, increased the extent of migration (Figure 3.4A).
Figure 3.2: Collectively migrating cohorts exhibit dynamic changes in shape, which are in phase and correlated with surrounding matrix deformations. (A) Confocal fluorescence image of a collectively migrating cohort after 40 hours with bead displacements superimposed (red). Contours of the migrating cohort in (A) from (B) 0 to 20 hours and from (C) 20 to 40 hours; darker colors represent later time points. (D) Plot of normalized cohort length (blue, dashed line) and projected area (red, solid line) for the cohort in (A). (E) Plot of normalized cohort length (blue, dashed line) and displacements (various colors, solid lines) of beads near (<50 µm) the migrating cohort. Also included is the displacement (black, dotted line) of one bead located far (>50 µm) from the migrating cohort. (F) Sample cross correlation plot comparing variations in cohort length to the displacement of a bead located near the migrating cohort. (G) Cross correlation coefficients comparing temporal change in cohort length to the displacement trajectories of fluorescent beads near the cohort and far from the cohort for two representative samples. Beads near cohort 1: n= 7; beads far from cohort 1: n= 11; beads near cohort 2: n= 7; beads far from cohort 2: n= 7. ***P<0.001. Scale bar, 50 µm.
Mechanical forces generate cellular deformations and propel cell movements during development and disease progression [174]. In addition to their physical roles, mechanical forces have signaling and regulatory functions [203]. To examine whether the tensile forces arising from the leading edge of the invading cohorts serve a role beyond facilitating physical translocation, we visualized signaling through FAK and p130Cas, both of which are activated downstream of integrins in response to mechanical force [204, 205, 206]. Immunofluorescence analysis revealed enhanced activation of both FAK and p130Cas within the extending cohorts; both localized to discrete matrix adhesions (Figure 3.3C). Consistently, disrupting cytoskeletal tension with blebbistatin abolished activation of FAK and p130Cas within the cohorts (Figure 3.3D).

Mechanical tension and FAK signaling have also been shown to promote the nuclear translocation of myocardin-related transcription factor (MRTF)-A [207]. Moreover, 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 [208]. Tensile forces generated during collective migration induce nuclear translocation of MRTF-A, its association with SRF, and subsequent transcription of SRF-target genes, regulating differentiation, proliferation, and motility [208]. MRTF-A is also required for cancer cell migration [209, 210] and branching morphogenesis of the *Drosophila* trachea [211] and is regulated by tension in mammalian epithelial cells [144, 212]. To test whether invasive collective migration of mammalian tissues is regulated by MRTF-A in a tension-dependent manner, we used immunofluorescence to characterize its localization (Figure 3.3E). MRTF-A was mainly nuclear in cells within the invading cohorts, whereas in quiescent tissue it was both nuclear and cytoplasmic (Figure 3.3F). To determine whether the nuclear translocation of MRTF-A was force-dependent, we altered tension in the cells by treating with blebbistatin (Figure 3.3G) or by controlling their distention using micropatterning (Figure 3.5A). Pharmacologically abolishing cytoskeletal tension significantly attenuated the nuclear translocation of MRTF-A (Figure 3.3H) as did reducing tension by restricting cell spreading (Figure 3.5A, B). Treating
the tissues with CCG1423, which blocks the interaction between MRTF-A and SRF [213], significantly impaired invasion (Figure 3.3I, J) as did shRNA-mediated depletion of MRTF-A (Figure 3.3K, L; Figure 3.5C). These data suggest that increased tension within the invasing cohort causes nuclear translocation of MRTF-A to promote collective migration.

Figure 3.3: Tensile forces drive collective migration by activating mechanically sensitive intracellular signaling and transcription factors. (A) Phase-contrast images showing collective migration from tissues treated with DMSO (control) or blebbistatin. (B) Quantification of cohort length from tissues treated with DMSO or blebbistatin. Mean ± s.e.m. of three replicates is shown, n= 60 tissues per group. **P<0.01. Immunofluorescence staining for FAK pY397 and phosphop130Cas in representative DMSO (C) and blebbistatin-treated (D) migrating cohorts. (E) Immunofluorescence staining for MRTF-A localization (green) and nuclei (red) in a representative tissue. (F) Quantification of the localization of MRTF-A (nucleus/cytoplasm) in tissues. Mean ± s.e.m. of three replicates is shown. Migrating
cohort (invasion): \( n = 14 \); quiescent body: \( n = 11 \). **P < 0.01. (G) Immunofluorescence staining for MRTF-A localization (green) and nuclei (red) in representative tissues treated with DMSO or blebbistatin. (H) Quantification of the localization of MRTF-A (nucleus/cytoplasm) in tissues treated with DMSO or blebbistatin. Mean ± s.e.m. of three replicates is shown. MRTF-A DMSO: \( n = 29 \); MRTF-A blebbistatin: \( n = 21 \); DAPI DMSO: \( n = 10 \); DAPI blebbistatin: \( n = 12 \). **P < 0.01. (I) Phase-contrast images showing collective migration from representative tissues treated with DMSO or CCG-1423. (J) Quantification of cohort length from tissues treated with DMSO or CCG-1423. Mean ± s.e.m. of three replicates is shown. Invading cohorts treated with DMSO: \( n = 80 \); invading cohorts treated with CCG-1423: \( n = 57 \). **P < 0.01. (K) Frequency maps showing collective invasion from 34 tissues transfected with scrambled shRNA (shScr) or shMRTF-A (two constructs). (L) Quantification of cohort length from tissues transfected with shScr or shMRTF-A. Mean ± s.e.m. of three replicates is shown, \( n = 50 \) tissues per group. ***P < 0.001. Scale bars, 50 \( \mu m \) (A,I,K), 25 \( \mu m \) (C,D,E,G).

3.2.4 Matrix alignment results in directional tracks for migration

It has been proposed that, during collective invasion through collagenous matrices, cells follow paths of least resistance created by proteolytic degradation and softening of the ECM [214]. To test for such a mechanism, we used confocal reflection microscopy (CRM) to visualize the structure of the matrix surrounding the invading cohorts (Figure 3.6B). We observed no apparent proteolytic remodeling ahead of the leading edge (Figure 3.7A-C; Figure 3.6A-C). Instead, we observed a different kind of matrix remodeling at these locations: collagen fibrils were compacted and aligned into parallel and highly directional tracks emanating from the invasive front and propagating over distances spanning ~100 \( \mu m \) from the tissue (Figure 3.7C). Measuring the angles of collagen fibrils revealed that those far from the tissue (Figure 3.7D) were distributed randomly (Figure 3.7E) while those ahead of the migrating cohort (Figure 3.7F) oriented preferentially in the direction of migration (Figure 3.7G). Imaging the matrix around primary organoids similarly revealed that collagen was compacted into dense and directionally oriented fibrils from the leading edge of extending branches (Figure 3.6E-G). Blocking cytoskeletal tension prevented collagen alignment ahead of the migrating cohorts (Figure 3.6I), suggesting that alignment was mediated by migration-generated tensile forces.
Figure 3.4: Tensile forces are required for collective migration and modulate activity of mechanosensitive proteins within the leading edge. (A) Quantification of cohort length from tissues treated with DMSO (control), Y27632, or LPA. Mean of three replicates is shown, n = 41 tissues per group. **P<0.01, ***P<0.001. Immunofluorescence staining for FAK pY397 in representative (B) Y27632-treated, (C) control, and (D) LPA-treated migrating cohorts. Immunofluorescence staining for phospho-p130Cas in representative (E) Y27632-treated, (F) control, and (G) LPA-treated migrating cohorts. Scale bars, 25 µm.
Figure 3.5: Nuclear translocation of MRTF-A is controlled by the tensional state of the cell. (A) Immunofluorescence staining for MRTF-A (green) and nuclei (red) in representative epithelial cells plated on adhesive islands of varying area (15 µm or 30 µm squares) to control the extent of cell spreading. Tension within the highly spread cells was dissipated by treating with blebbistatin. (B) Quantification of the localization of MRTF-A (nucleus/cytoplasm) in cells on 15 µm or 30 µm squares treated with DMSO (control) and cells on 30 µm squares treated with blebbistatin. Mean ± s.e.m. of three replicates is shown, n=5 cells per condition. **P<0.01. (C) Transcript levels of MRTF-A in epithelial cells transfected with scrambled control shRNA (shScr) and shRNA against MRTF-A (shMRTFA, two constructs). Mean ± s.e.m. of three replicates is shown. **P<0.01.
Figure 3.6: Tensile forces remodel collagen at the leading edge of migrating cells. (A) Confocal fluorescence image showing a representative collectively migrating epithelial tissue labeled with DiI (red). (B) Confocal reflection image showing collagen structure (green) around migrating cohort in (A). (C) Merged image of (A) and (B). (D) Phase-contrast images of a representative mammary organoid. (E) Confocal reflectance image showing the structure of collagen surrounding the primary mammary organoid in (D). (F) Merged image of (D) and (E). (G) High magnification image of a region in (F) showing aligned matrix at the leading edge of a migrating cohort. (H) Phase-contrast image of a representative cluster of collectively migrating epithelial cells after treatment with blebbistatin. (I) Confocal reflection image showing the structure of collagen surrounding the cluster of collectively migrating mammary epithelial cells in (H). Scale bars, 50 µm.
The generation of physiologically functional tissue geometries during epithelial morphogenesis requires tight spatial guidance of collective cell movements. Hence, it is necessary to determine the guidance cues that initiate and propel movement as well as those that confer and maintain directionality. Classically, guidance roles have been attributed to soluble cues, including growth factors and various chemokines [215, 216, 217]. Recently, however, long-range transmission of mechanical signals has been proposed to independently guide collective cell migration in the context of epithelial tubulogenesis [218, 219]. Cells migrate more efficiently through directionally aligned fibrillar matrices than randomly oriented ones [119, 220]. Therefore, we postulated that tension-mediated alignment ahead of the invasive front facilitates further collective migration and provides directionality to the movement. To test these hypotheses, we incorporated epithelial tissues into regions of pre-aligned ECM (of length scales similar to those ahead of the leading edge of migrating cohorts). Mechanical strains...
generated by tissues of non-circular geometries are non-uniformly distributed within the surrounding matrix (Figure 3.8A) [165, 166]. CRM revealed that the ECM was preferentially remodeled and aligned in regions experiencing high strains (Figure 3.8B). In contrast, tissues of circular geometry experienced no spatial variations in the structure or alignment of the surrounding ECM (Figure 3.8C, D). Consistently, collective invasion from these circular tissues occurred without directional preference (Figure 3.8E, F). However, when rectangular and circular tissues were juxtaposed to align the ECM at specific locations around the latter (Figure 3.8G, H), a directional bias emerged: cohorts from the circular tissues migrated preferentially in the direction of aligned fibrils (Figure 3.8I). Furthermore, cohorts migrating along aligned fibrils were longer and contained more cells than did those migrating through randomly oriented matrix (Figure 3.8J). To rule out chemoattraction as a possible explanation for the migration bias, we altered the relative configuration such that rectangular tissues no longer aligned the ECM surrounding the circular tissues (Figure 3.8K, L). The migration bias disappeared (Figure 3.8M, N), confirming that the directional cue was provided by ECM alignment and not soluble factors. These data indicate that matrix alignment plays two roles during collective migration: it increases the efficiency of migration and spatially directs migrating cohorts.
Figure 3.8: Matrix alignment spatially directs collective migration. (A) Heat map showing matrix deformation around rectangular tissues. (B) Confocal reflection image showing the structure of collagen surrounding a rectangular tissue labeled with DiI (red) prior to collective migration. Also shown are high magnification images of regions of fibril alignment near the tips of the tissue. (C) Schematic and (D) confocal reflection image of collagen surrounding a circular tissue prior to collective migration. (E) Rose plot showing the angles of collective migration from 66 circular tissues. (F) Frequency map representing collective migration from the tissues in (E). (G) Schematic and (H) confocal reflection image of collagen surrounding a circular tissue exposed to regions of fibril alignment prior to collective migration. (I) Rose plot showing the angles of collective invasion from 83 tissues in the configuration shown in (G). (J) Frequency map representing collective migration from the tissues in (I). (K) Schematic and (L) confocal reflection image of collagen matrix surrounding a circular tissue.
proximal to rectangular tissues, but not exposed to regions of preferential fibril alignment prior to collective migration. (M) Rose plot showing the angles of collective invasion from 53 tissues in the configuration shown in (K). (N) Frequency map representing collective migration from the tissues in (M). Scale bars, 50 μm.

3.3 Discussion

Together, these results reveal an essential and multifaceted role for endogenous mechanical forces during collective migration through 3D fibrillar matrices. We characterized and made quantitative estimates of the tensile forces that arise during this process, the nature and existence of which had only been inferred thus far [191]. We showed that migrating collectives propel themselves through fibrillar matrices by pulling on impeding fibers. Furthermore, our data suggest that this physical mode is a general migration strategy, as functionally normal epithelial cells, cancer cells, and primary organoids moved collectively using a similar mechanism. Ours is the first study to examine the temporal and spatial dynamics of physical forces exerted by a migrating cohort fully embedded in a 3D matrix. Importantly, our data show that the cohort does not exert continuous tensile force on the surrounding ECM. Instead, the multicellular collective frequently pauses and apparently releases its grip on the matrix or relaxes its hold as it extends. The length of the migrating cohort is not monotonically increasing; rather, we observed periods of extension followed by periods of retraction. Protrusion and retraction have also been reported in the collective migration of Drosophila border cells [221], although these motions were not correlated with deformations in the surrounding microenvironment in that study.

Importantly, the forces that arise during collective migration have both physical and signaling roles. In addition to providing ‘grip’ and ‘pull’ to enable translocation, these tensile forces regulate the efficiency and direction of migration by conditioning both intracellular components and the ECM. The mechanosensitive focal adhesion proteins FAK and p130Cas, both regulators of cytoskeletal tension, motility, and invasiveness, were preferentially activated in a force-dependent manner within the invasive front of the migrating cohorts. These
results are consistent with studies in 2D epithelial monolayers in which cytoskeletal tension in regions of high curvature was found to be important for leader cell formation during collective migration [222]. We also found that mechanical force regulated nuclear localization, and likely transcriptional activation of MRTF-A, which was required for collective migration. Notably, MRTF-A regulates genes that encode cytoskeletal and adhesion proteins involved in the force-generating machinery of the cell [209]. Accordingly, it is possible that intracellular forces and MRTF-A engage in a dialogue of positive feedback, ultimately increasing the efficiency of collective migration. It will be interesting to determine how the temporal changes in force discussed above correlate with the dynamics of molecular signaling at cell-matrix and cell-cell junctions as well as downstream of Rho and Rac small GTPases.

A recent study reported that E-cadherin adhesions between border cells and nurse cells in the Drosophila ovary participate in a positive feedback loop with Rac and actin assembly to stabilize directed collective migration [221].

Mechanical forces facilitate collective migration not only by influencing the cells themselves but also by priming the surrounding ECM. We found that the matrix preceding a migrating cohort is remodeled into parallel fibrils in a process requiring mechanical force. Proteolysis may be required for collective migration as a means to accommodate the growing structure [223] rather than creating paths along which migration occurs though pericellular proteolysis may assist in ECM remodeling. Others have shown that collagen gels can be aligned via mechanical strain even in the absence of cells [224]. In our system, these directionally aligned collagen fibrils increase collective migration efficiency by providing a path for persistent motility. Our findings are consistent with models suggesting that collective migration follows paths of least resistance [214]. However, our data show that these paths are primarily generated via physical force and that tissue geometry plays a role in the localization of these forces and subsequent ECM alignment. Moreover, our findings are in accord with those of Liphardt and colleagues, who have shown that cells in mammary acini can mechanically align and concentrate surrounding ECM fibers over long distances [225].
Consistently, contractility-mediated local collagen reorganization at the tumor-stroma interface has been shown to promote cancer cell invasion [11, 119, 220, 226]. A recent study demonstrated enhanced collagen alignment proximal to the terminal end buds of the mouse mammary gland and suggested that these patterned collagen fibers orient the branching mammary epithelium [227]. Despite evidence showing that Rho-mediated contractions are required for collagen remodeling [220, 227], it is unclear how matrix alignment is restricted to the leading edge of a globally contracting tissue. We found that the tensile forces driving migration are highly localized (Figure 3.1E, G, I, K) and responsible for generating the restricted patterns of matrix alignment. The spatial distribution of forces is in turn controlled by the geometry of the cohort; by engineering tissues of tubular geometry, we were able to restrict both contractile forces and collagen alignment to a specific region of the tissue (Figure 3.8A, B). The resulting narrow (50-100 µm) strips of aligned collagen successfully controlled the directionality of migrating cohorts. An important implication of these findings is that the geometries of tissues generated through collective migration may be self-referential; the initial geometry dictates the emergent geometry by mechanically controlling the pattern of matrix remodeling.

Notably, although normal epithelial cells and invasive cancer cells migrated collectively via similar physical mechanisms, we observed striking differences in the spatial organization of the forces and resulting matrix remodeling (Figure 3.1F-K). In particular, whereas collective migration of non-neoplastic cells generated highly restricted and directional force fields and matrix alignment, the forces produced by migrating cancer cohorts appeared to be diffuse and delocalized. Comprehensive understanding of the differences between collective cell movement during morphogenesis and cancer progression will require dynamic spatiotemporal mapping of the force fields and matrix remodeling associated with the two types of processes in vivo.
3.4 Materials and methods

3.4.1 Cell culture and reagents

Functionally normal EpH4 mouse mammary epithelial cells [164] were cultured in 1:1 DMEM:F12 supplemented with 2% fetal bovine serum (FBS; Atlanta Biologicals), 5 µg/ml insulin, and 50 µg/ml gentamicin (Sigma). MDA-MB-231 human breast cancer cells (ATCC) were cultured in 1:1 DMEM:F12 supplemented with 10% FBS and 50 µg/ml gentamicin. Primary mammary epithelial organoids were prepared from 8-week-old CD1 mice (Charles River) as described previously [228]. Isolated organoids were resuspended in 1:1 DMEM:F12 supplemented with 10% FBS, 5 ng/ml EGF, insulin/transferrin/sodium selenite (ITS; Sigma), and penicillin/streptomycin (Sigma), and embedded immediately in collagen gels. Cells were maintained in a 37°C incubator with 5% CO₂. The following reagents were used at the concentrations indicated: blebbistatin (12.5 µM; Sigma), CCG1423 (10 µM; Cayman Chemicals), Y27632 (20 µM; Tocris), lysophosphatidic acid (LPA; 10 µg/ml; Cayman Chemicals) and were added to the medium 1-4 hours after tissues began collective migration.

3.4.2 Microfabricated tissues and 3D culture models

3D epithelial tissues were constructed as described previously [229, 230]. Briefly, neutralized non-pepsinized native type I collagen (BD Biosciences) was gelled at 37°C against a stamp of PDMS (Sylgard 184, Ellsworth Adhesives) to generate micrometer-scale cavities of defined geometry. Mammary epithelial cells were allowed to settle within the cavities, and a second layer of collagen was placed on top of the gel. Medium supplemented with HGF (10 ng/ml) was added to the samples ∼20 hours later, after which the tissues underwent collective migration for 24-48 hours. Matrix deformations during migration were visualized by incorporating 1-µm diameter fluorescent polystyrene beads (Invitrogen) in the collagen solution (∼4x10⁸ beads/ml). Clusters of mammary epithelial or tumor cells were prepared.
by shaking overnight (170 rpm at 37°C for 14 h) in the presence of 0.083% (w/v) pluronic F108 (BASF). Clusters of cells 100 µm in diameter were collected by brief centrifugation (200 rpm for 1 min) and embedded within 6 mg/ml of type I collagen as described previously [228]. A cell-free layer of collagen was included beneath the layer containing clusters. Medium supplemented with HGF (10 ng/ml) was added to the samples.

3.4.3 Immunofluorescence analysis

Samples were washed in PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Samples were permeabilized twice for 10 min in 0.5% Igepal Ca-630 and incubated in 0.1% Triton X-100 in PBS for 15 min. Samples were blocked overnight at 4°C in 10% goat serum in PBS (PBS-S) followed by overnight incubation at 4°C in primary antibody against FAK pY397 (Invitrogen 44-624G), phospho-p130Cas (Cell Signaling 4015S), or MRTF-A (Sigma HPA030782) at 1:100 dilution in PBS-S. Samples were washed with PBS and incubated in secondary antibody at 1:1000 in PBS-S overnight at 4°C.

3.4.4 Constructs, transfection, and transduction

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 using Fugene HD (Roche). To visualize collective movements, epithelial cells were transduced with a recombinant adenovirus encoding LifeAct-GFP [231] and/or H2B-mCherry (Vector Biolabs) at an MOI resulting in >99% transduction efficiency.

3.4.5 Quantitative image analysis

The length of collectively migrating cohorts was measured from the edge of the original tissue to the tip of the migrating cohort using ImageJ software (NIH). The levels of nuclear and cytoplasmic MRTF-A in confocal slices of stained samples were quantified by measuring
the signal intensity in the two compartments using ImageJ. Frequency maps of collective migration were created using binarized images of \( \sim 50 \) tissues of identical initial geometry. These were stacked with Scion Image software to obtain a pixel frequency map and color-coded in Adobe Photoshop.

3.4.6 Real-time microscopy and measurement of matrix displacements

Timelapse 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\(_2\). Fluorescent beads and LifeAct-GFP labelled cells were imaged simultaneously. Confocal stacks (390 \( \times \) 390 \( \times \) 100 \( \mu \)m, spaced 1 \( \mu \)m in the z-direction) were acquired using a Plan Apo 20 \( \times \) 0.4 NA objective every 2 hours beginning at 24 hours after initial microfabrication and addition of HGF for a total of 20-48 hours. To measure matrix deformations at a given time during the migration process, bead positions were recorded before and after lysing the collectively migrating tissue using 0.1% (w/v) of Triton X-100. 3D and bead displacements were extracted using the Autoregressive Motion tracking algorithm in the image analysis software Imaris® (Bitplane). The displacement gradient matrix for finite strains was used to calculate tissue-induced strains within the collagen gel:

\[
\varepsilon_{ij} = \frac{1}{2} \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} - \frac{\partial u_k}{\partial x_i} \frac{\partial u_k}{\partial x_j} \right)
\] (3.1)

3.4.7 Mechanical properties and constitutive model of collagen gels

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 to mimic experimental conditions and to prevent the collagen from drying. To rigorously compute the mechanical stresses exerted by the migrating cohort, the anisotropic, viscoelastic behavior of the collagen matrix must be considered. In this study, however, we were not concerned with the absolute magnitude of the computed stresses; rather, we sought to estimate the spatial and temporal distributions of mechanical stress with respect to the migrating cohort and (as a first approximation) made several simplifying assumptions. Hence, Hooke’s law for isotropic materials was used to describe the constitutive behavior of the collagen gels during tissue-induced deformation:

\[
T_{ij} = \frac{1}{2}[\lambda \varepsilon_{kk} \delta_{ij} + 2\mu \varepsilon_{ij}]
\]  
(3.2)

\[
\lambda = \frac{\nu E}{(1+\nu)(1-2\nu)}
\]  
(3.3)

\[
\mu = \frac{E}{2(1+\nu)}
\]  
(3.4)

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 [232].

### 3.4.8 Epithelial tissue surface reconstruction and mesh generation

The surface of the branched epithelium at a snapshot in time (24 hours after branch induction) was reconstructed from 3D confocal stacks of LifeAct-GFP-transduced cells. Image segmentation was performed manually in ImageJ to define the cellular portion of the 3D stack. 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.
### 3.4.9 Calculation and reporting of mechanical stress

The equations of motion, the displacement gradient matrix (Equations 3.1), and Hooke’s law for isotropic materials (Equations 3.2-3.4) were used to calculate the Cauchy stress tensor throughout the domain, as described previously [165]. The boundary conditions were as follows: displacements at the epithelium-matrix interface were interpolated from experimentally measured bead displacement values. A displacement of zero was assumed 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:

\[
t_i = \sum_j T_{ji} n_j \quad (3.5)
\]

\[
|t| = \sqrt{t_1^2 + t_2^2 + t_3^2} \quad (3.6)
\]

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 \(|t|\) is the magnitude of the traction vector.

### 3.4.10 Correlation analysis

Correlations between bead displacements and length variations of a migrating cohort were determined using the sample cross covariance function, which indicates the covariance between discrete sets of data collected over time and determines whether there is a lag between the two time series. For two time series \(x_{1,t}\) and \(x_{2,t}\), the lag \(k\) cross-covariance is estimated as:

\[
c_{x_{1}x_{2}}(k) = \begin{cases} 
\frac{1}{T} \sum_j (x_{1,t} - \bar{x}_1)(x_{2,t+k} - \bar{x}_2); & k = 0, 1, 2... \\
\frac{1}{T} \sum_j (x_{2,t} - \bar{x}_2)(x_{1,t-k} - \bar{x}_1); & k = 0, -1, -2... 
\end{cases} \quad (3.7)
\]
Here, $\bar{x}_1$ and $\bar{x}_2$ are the sample means of the time series. The cross correlation coefficient is:

$$r_{x_1x_2}(k) = \frac{c_{x_1x_2}(k)}{s_{x_1}s_{x_2}}; k = 0, \pm 1, \pm 2...$$

(3.8)

where $s_{x_1}$ and $s_{x_2}$ are the sample standard deviations of the time series. Lag $k$ cross-covariances and cross correlation coefficients were calculated between the length variations of a migrating cohort and the displacements of beads within the surrounding ECM that were both adjacent to (<50 µm) and far from (>50 µm) the extending cohort.

### 3.4.11 Confocal reflection microscopy

The fibrillar structure of the collagen was visualized using a Leica SP5 laser-scanning confocal microscope as described previously [165]. Epithelial tissues were fabricated as described above; cells labeled with DiI were embedded in a matrix composed of type I collagen and Matrigel (BD Biosciences) in a 1:4 ratio. Collagen matrices were illuminated with an Argon laser (488 nm) and imaged in reflection mode using a 20× or 63× oil-immersion objective. Images of the collagen surrounding tissues (prior to migration) were taken at a z-position corresponding to the middle of the tissue.

### 3.4.12 Quantifying collagen fibril alignment

Collagen fibril alignment was quantified by measuring the orientation of pixel intensity gradients in subregions of the confocal reflection images. The results were displayed in a circular histogram [233]. To compare these fibril orientations ($\alpha_i$) with branch angles ($\varphi$) in cultured mammary organoids, we calculated the angle difference $\theta_i = \varphi - \alpha_i$ in the neighborhood of extending branches, where $\theta = 0$ indicates fibers precisely aligned with an extending branch. As a control, fibril orientations ($\alpha_i$) were also computed for regions of the collagen gel away from cultured organoids.
3.4.13 Statistical analysis

Results were analyzed in GraphPad Prism (GraphPad Software). The two-tailed Student’s $t$-test and two-tailed Kruskal-Wallis test with Dunn’s multiple comparison post-test were used where appropriate. The alpha level was set at 0.05 for all statistical tests. The normality of data was confirmed prior to use of parametric tests.
Chapter 4

Dynamics and Mechanisms of Cell-Induced Alignment of Fibrous ECM

4.1 Introduction

Cells within tissues are surrounded by the ECM, which is an important structural component in tissues that contains a variety of chemical and mechanical cues that are recognized by cells. Cells can sense the rigidity of the ECM, and the topography of the polymer network dictates the number and spatial distribution of cell-matrix attachments [52, 234, 235]. Additionally, individual ECM protein fibers can serve as potential paths for cell migration. Particularly in the context of cancer cell invasion, alignment of the ECM perpendicular to the boundary of a tumor is thought to precede invasion with the aligned fibers providing tracks that guide migration [119, 236]. Moreover, previous results from our laboratory revealed that the alignment of collagen fibers directly influences the course and extent of collective cell migration in non-metastatic cells [167]. An aligned matrix has also been shown to improve the migration efficiency of breast cancer cells by increasing directional persistence along
aligned collagen fibers [236]. Cell-induced alignment of ECM fibers therefore appears to be an important component of the migration process preceding translocation. However, little is known about the dynamics of cell-induced alignment or which cellular processes drive the alignment of adjacent fibers in 3D. It is also unclear how the timescale for alignment compares to the timescale for migration.

Naturally, cell-matrix interactions are crucial for the ECM alignment process. We have previously demonstrated that cell-derived forces, such as contractility via Rho signaling, drive collective migration through 3D matrices; alignment of the pericellular matrix can influence both the direction and the extent of migration [167]. As alignment generally seems to precede migration, we hypothesized that cellular contractility also plays a role in matrix alignment prior to cell movement. Consistently, we had found that inhibiting cytoskeletal tension prevented collagen alignment in front of collectively migrating cells, suggesting that cellular contractility mediates matrix alignment. Others have found that collagen fibers can be aligned by mechanical strain alone with the majority of fibers being aligned under 30% strain [236]. Nonetheless, matrix remodeling via pericellular proteolysis could also be important for alignment in vivo. For example, proteolytic remodeling of the ECM by the membrane-bound MMP14 can influence the alignment of matrix fibers during both individual and collective cell migration [13]. We sought to characterize the dynamics of cell-induced matrix alignment and to determine whether contractility, proteolysis, or both are necessary for alignment.

4.2 Results

4.2.1 Cells exert strain to align surrounding matrix fibers

We took advantage of a microfabrication-based approach to generate hundreds of identically shaped 3D epithelial tissues embedded within an ECM gel and used this system to investigate the dynamics of matrix alignment. From our previous work using CRM to image the
structure of the collagen matrix [167], we found that cells seeded to form 100 µm-diameter circular tissues align the ECM radially around the tissue within 24 hours (Figure 3.8D). To determine a more precise timeframe for alignment, we visualized the matrix surrounding tissues of circular cross-sectional geometry at various time points between seeding (0 hours) and 24 hours and quantified the alignment of fibers adjacent to the tissues relative to the tissue surface (Figure 4.1). We found that approximately half of the collagen fibers that were located within 100 µm of the tissues became aligned (i.e. oriented approximately perpendicular to the tissue surface) within 6 hours. This alignment profile did not change significantly between 6 and 24 hours, suggesting that the cells rapidly aligned their surrounding matrix within a few hours after seeding. To further quantify changes in fiber alignment over time, we examined temporal variations in the percentage of unaligned ECM fibers within a 100 µm radius of the tissues. This parameter exhibited an exponential decay-like trend over time, reaching an asymptote of ~50% unaligned fibers after 6 hours (Figure 4.2A). Using these data, we computed a characteristic timescale for fiber alignment which we called the alignment half-life \( t_{1/2} \). For mammary epithelial tissues of circular geometry, the alignment half-life was approximately 3.1 ± 0.2 hours, suggesting that cells rapidly remodel their surrounding ECM.

The cells are initially rounded upon seeding (Figure 4.1A) and lack attachments to the surrounding matrix. Immediately thereafter, the cells begin to adhere to each other and to the surrounding matrix; and the tissue contracts inwards (Figure 4.2). By measuring the change in radius of the tissues, we approximated the strain that the cells exert on their adjacent matrix as a function of time (Figure 4.2B). We found that the radius of circular tissues shrinks by approximately 33.9 ± 0.3% within the first 6 hours, suggesting that the tissue imposes a radial strain of ~34% on the surrounding matrix. The strain did not increase between 6 hours and 24 hours. The trend of cell-induced strain over time is concordant with the decay of unaligned fibers surrounding the tissues; both curves reach a plateau by approximately 6 hours. To visualize the dynamics of matrix alignment around
Figure 4.1: Cells in engineered tissues rapidly align adjacent collagen fibers. Confocal images of circular epithelial tissues (red) and their surrounding collagen matrix (white) taken at (A) 0 hours, (C) 2 hours, (E) 4 hours, (G) 6 hours, and (I) 24 hours after tissue seeding. Corresponding fiber alignment relative to the tissue surface for (B) 0 hours, (D) 2 hours, (F) 4 hours, (H) 6 hours, and (J) 24 hours after tissue seeding. Scale bars, 50 µm.
individual tissues, we used timelapse imaging. Consistent with our fixed time point data, timelapse analysis revealed that tissues contract within a few hours (Figure 4.2C-E) while simultaneously radially aligning the surrounding matrix. These results suggest that the mechanism for fiber alignment is cell-induced strain on the matrix as a result of tissue contraction.

![Figure 4.2](image_url)

Figure 4.2: Tissues contract inwards, imposing a strain that aligns the ECM. (A) Percentage of unaligned fibers over time. (B) Radial strain exerted by cells over time. Mean ± S.E.M. of three replicates is shown. Timelapse confocal images of a representative circular epithelial tissue (red) and its surrounding collagen matrix (white) taken at (C) 0 hours, (D) 2 hours, and (E) 4 hours after tissue seeding. Yellow dotted circle indicates initial geometry and white arrows indicate collagen fibers aligning adjacent to the tissue surface. Scale bars, 50 μm.
4.2.2 Cellular contractility drives matrix alignment

Given that we observed a decrease in tissue radius concurrent with matrix alignment and that mechanical strain in the absence of biochemical factors and degradation is sufficient to align collagen fibers [236], we hypothesized that cellular contractility is the driving force behind cell-induced matrix alignment. We tested this hypothesis by manipulating contractility pharmacologically. Upon treatment with Rho kinase inhibitor, Y27632, the circular tissues were unable to radially align their surrounding matrix even after 24 hours (Figure 4.3A). Treatment with the myosin ATPase inhibitor, blebbistatin, had a similar effect. DMSO control samples aligned the matrix on a similar timescale to untreated tissues (3.1 ± 0.3 hours). In the presence of Y27632, cells exerted less strain on the surrounding matrix over 6 hours (7.5 ± 1.1%) compared to DMSO controls (33.5 ± 0.5%) (Figure 4.3B). Similarly, treatment with blebbistatin resulted in reduced strain (9.0 ± 0.7%). Conversely, increasing contractility via treatment with a Rho-activator, LPA, decreased the alignment half-life to 2.3 ± 0.1 hours compared to controls (3.6 ± 0.5 hours) (Figure 4.4A). LPA-treated samples reached a maximum strain of 35.0 ± 0.7% which was similar to DMSO controls (33.8 ± 0.2%) except that LPA samples reached the plateau within 5 hours (Figure 4.4B). These data suggest that cellular contractility mediated by the Rho/ROCK pathway is necessary for pericellular matrix alignment. Varying cytoskeletal tension alters the dynamics of matrix alignment, with increasing contractility resulting in increased strain rate and a shorter time to alignment.

4.2.3 Proteolysis is not required for matrix alignment

Previous studies have suggested the importance of matrix remodeling by MMPs during cell migration; therefore, we sought to determine whether matrix alignment was influenced by MMP-dependent proteolysis prior to migration. To determine whether MMP activity is necessary for matrix alignment, we treated tissues with the broad-spectrum MMP inhibitor, GM6001, and found that upon treatment alignment half-life (3.6 ± 0.3 hours) was essentially
Figure 4.3: Inhibiting contractility inhibits fiber alignment and decreases cell-induced strain. (A) Percentage of unaligned fibers over time for tissues treated with Y27632 (dashed line) or Blebbistatin (dotted line) compared to DMSO controls (solid line). (B) Radial strain exerted by cells over time for tissues treated with Y27632 (dashed line) or Blebbistatin (dotted line) compared to DMSO controls (solid line). Mean ± S.E.M. of three replicates is shown.

The same as that of DMSO controls (3.6 ± 0.5 hours) (Figure 4.5A). The maximum strain in GM6001 samples was 33.5 ± 1.1% which was again similar to DMSO controls (33.2 ± 0.5%) (Figure 4.5B). These results suggest that MMP activity is not required for alignment of the pericellular ECM. Others have shown that MMP activity is, however, required for efficient migration, particularly in regions of dense ECM where the small pore sizes in the matrix prohibit cells from passing through [237].
4.2.4 Cancer cells are slower to align their matrix compared to non-neoplastic cells

Cancer cells that have undergone EMT have been shown to be more contractile than their more epithelial non-neoplastic counterparts [238]. As we observed that pharmacologically increasing contractility resulted in faster matrix alignment, we hypothesized that more contractile cells would align their surrounding matrix with a shorter characteristic timescale. To test this hypothesis, we created 100 µm-diameter circular tissues using MDA-MB-231 breast cancer cells, which are mesenchymal in morphology and highly invasive in culture [46]. Similar to tissues of normal mammary epithelial cells, MDA-MB-231 tissues aligned approximately 50% of their surrounding ECM fibers within 24 hours after seeding (Figure 4.6A).
Figure 4.5: Inhibiting MMP activity does not affect alignment dynamics. (A) Percentage of unaligned fibers over time for tissues treated with GM6001 (dashed line) or DMSO control (solid line). (B) Radial strain exerted by cells over time for tissues treated with GM6001 (dashed line) or DMSO control (solid line). Mean ± S.E.M. of three replicates is shown.

However, MDA-MB-231 cells were slower to align their surrounding matrix, with an alignment half-life of 5.0 ± 0.5 hours. In addition, MDA-MB-231 tissues imposed a lower radial strain (23.4 ± 0.3%) on the matrix over the course of 24 hours (Figure 4.6B), which could contribute to the increased time to ECM alignment. We also noted that these cells do not form many intracellular connections, therefore the tissues did not contract inwards as uniformly as those comprised of normal cells. These results suggest that, whereas MDA-MB-231 cells are individually contractile, they do not impose the same level of strain as non-malignant epithelial cells when cultured as multicellular tissues.
Figure 4.6: Cancer cells take longer to align their surrounding matrix. (A) Percentage of unaligned fibers over time for tissues comprised of MDA-MB-231 cells. (B) Radial strain exerted by cells over time for tissues comprised of MDA-MB-231 cells. Mean ± S.E.M. of three replicates is shown.

4.3 Discussion

Collagen alignment is associated with increased efficiency of cell migration [236]. In the context of cancer invasion, ECM alignment has also been associated with enhanced metastasis and poor prognosis [119, 239]. While cell movement along aligned matrix fibers has been frequently observed, the process by which the fibers are initially aligned remains poorly understood. In this work, we used a 3D culture model to determine the dynamics and underlying mechanisms by which tissues align their surrounding matrix prior to migration. We found that tissues rapidly aligned their surrounding matrix with the majority of the fibers adjacent to the tissue surface oriented perpendicularly within 6 hours. Cellular contractility was the driving force for matrix alignment, with MMP proteolysis dispensable.
These data show evidence that the timescale for matrix alignment is significantly faster than the timescale for migration. We previously observed collective migration from these engineered tissues to occur over 24-48 hours [167]. Interestingly, we have also estimated that cell-induced matrix deformation and collective migration require approximately the same magnitudes of force [165, 167]. However, in the case of migration, it appears that these forces must be exerted over longer periods of time in order to result in cell translocation once fibers are aligned. Given that alignment appears to precede migration and provide directionality to cell movements, the ability of cells to quickly align the matrix may be beneficial in vivo, enabling them to make rapid changes in the direction of migration in accordance with changing environmental cues.

As we had previously observed that physical force generation via cytoskeletal tension is the driving mechanism behind collective migration, it was not surprising that contractility is also necessary for ECM alignment and that the underlying mechanisms behind the two processes are linked. Consistent with our results, others have also implicated ROCK-mediated contractility in matrix contraction and fiber displacement [220, 227, 240, 241]. The result that MMP activity has little to no effect on alignment indicates that cell-induced matrix alignment is primarily a mechanical process by which fibers in the ECM network are strained by cells. Accordingly, others have shown using finite element simulations that tension in the absence of degradation is sufficient to align collagen fibers [242]. Furthermore, there are several experimental examples of collagen fibers being aligned using mechanical forces alone, such as through fluid flow or stretching devices that apply mechanical strain [224, 236]. The extent of alignment in these cases is determined by the amount of strain exerted on the collagen gels. Likewise, in our system, the extent of matrix alignment around multicellular tissues appears to be determined primarily by the amount of tensile force that the cells are collectively able to generate via contractility in order to strain their surrounding fiber network.
Paradoxically, the contractility of individual cells does not necessarily correlate with increased matrix alignment, even though it has been suggested that the range of cell-matrix force transmission increases with increasing contractility [242]. We found that MDA-MB-231 breast cancer cells were slower to align their surrounding matrix than normal mammary epithelial cells. One possible explanation is that the collective contractility of multiple cells is important; increasing contractility on the level of individual cells comprising a multicellular tissue does not necessarily translate to faster alignment around the tissue as a whole. In fact, our results suggest that cell-cell contacts, such as those mediated by E-cadherin, may be more important than individual cellular contractility to align the matrix around multicellular tissues. Intercellular connections likely enable force propagation across neighboring cells [243], resulting in greater tension applied at cell-matrix adhesions and consequently faster pericellular matrix alignment.

The correlation between fiber alignment and cell migration underlines the importance of determining the mechanisms by which cells interact with their surrounding ECM, as the results have wide-ranging implications for both normal and pathological phenomena. Rho-mediated contractility in particular appears to be required for both alignment of the ECM and migration along matrix fibers in several different cell types [236, 244, 245], confirming the role of mechanotransduction and physical forces in a range of morphogenetic processes. Furthermore, it seems that the coordination of cell-cell adhesions enables the transmission of cell-derived forces over tissue-level length scales. When combined with cell-matrix adhesions, cell-derived forces can in turn yield larger ECM network deformations and faster migration speeds. Both of these interactions are important in vivo for large-scale tissue deformations, such as morphogenetic movements that occur during development [246]. In the context of cancer invasion, our results suggest that it is beneficial for cancer cells to retain some level of epithelial characteristics and cell-cell contacts in order to propagate cytoskeletal tension across multiple cells and rapidly align their surrounding ECM prior to invading. Further investigation of the physical interplay between cells and their ECM will continue to provide
insight as to how biomechanics contribute to collective migration in development and disease contexts.

4.4 Materials and methods

4.4.1 Cell culture and reagents

Functionally normal EpH4 mouse mammary epithelial cells [164] were cultured in 1:1 DMEM:F12 medium supplemented with 2% fetal bovine serum (FBS; Atlanta Biologicals), 5 µg/ml insulin, and 50 µg/ml gentamicin (Sigma). MDA-MB-231 human breast cancer cells (ATCC) were cultured in 1:1 DMEM:F12 supplemented with 10% FBS and 50 µg/ml gentamicin. Cells were maintained in a 37°C incubator with 5% CO₂. The following reagents were used at the concentrations indicated: blebbistatin (12.5 µM; Sigma), Y27632 (20 µM; Tocris), lysophosphatidic acid (LPA, 10 µg/ml; Cayman Chemicals), GM6001 (40 µM; Calbiochem).

4.4.2 Microfabricated 3D tissues

3D epithelial tissues were constructed as described previously [229, 230]. Briefly, acid-extracted bovine type I collagen (Koken) at a final concentration of 4 mg/mL was mixed with Matrigel (BD Biosciences) in a 1:4 ratio and gelled at 37°C against a stamp of PDMS (Sylgard 184, Ellsworth Adhesives) to generate micrometer-scale cavities of defined geometry. Mammary epithelial cells were allowed to settle within the cavities, and a second layer of the collagen and Matrigel mixture was placed on top of the gel.

4.4.3 Confocal reflection microscopy

The fibrillar structure of the collagen was visualized using a Nikon A1 laser-scanning confocal microscope in reflection mode. Cells were transduced with AdLifeAct-mRuby at an MOI
resulting in >99% transduction efficiency. Collagen matrices were illuminated with an Argon laser (488 nm) and imaged in reflection mode using a 40× oil-immersion objective. Image stacks (319 µm × 319 µm × 30 µm, spaced 2 µm in the z-direction) were acquired for each engineered tissue and the surrounding collagen fibers.

4.4.4 Quantifying fiber alignment

Confocal z-stacks of the collagen fibers surrounding the epithelial tissues were projected onto a single image based on maximum pixel intensity to visualize the structure of the network. The engineered tissues were then segmented and linearized in ImageJ in order to quantify collagen alignment relative to the surface of the tissues. Collagen fibril angles were quantified by measuring the orientation of pixel intensity gradients of the confocal reflection images as described previously [233].

4.4.5 Real-time microscopy

Timelapse movies of engineered tissues between 0 and 8 hours after seeding were collected using a Nikon A1 laser-scanning confocal microscope in reflection mode fitted with a humidified environmental chamber held at 37°C and 5% CO₂.
Chapter 5

IFP Regulates Collective Invasion in Engineered Breast Tumors via EMT Marker Expression

This chapter is adapted from the following publication:


5.1 Introduction

During the transformation of healthy tissue into invasive cancer, the cellular microenvironment undergoes several physical changes [247]. As solid tumors grow, their lymphatic vessels collapse due to increased mechanical compression from the presence of extra cells [248]. The non-functioning intra-tumoral lymphatic system impairs drainage from the tumor, and hyperpermeable blood vessels lead to an accumulation of fluid and plasma macromolecules within the interstitium [248]. As a result, the IFP within tumors tends to be dramatically
elevated, resulting in localized interstitial hypertension [248, 249]. In human and animal tumors, IFP rises steeply within the tumor periphery and plateaus at values as high as 50 mm Hg (compared to 0 mm Hg in normal tissue) [176, 184, 250, 251, 252, 253], resulting in an outward flow of fluid from the tumor core [175, 254]. Cellular responses to this physical abnormality result in increased tumorigenic potential. High IFP has been associated with poor prognosis [177] and increased lymph node metastasis [176] in cervical cancer. The molecular mechanisms underlying the effects of IFP on tumor progression and invasion, however, remain unclear.

3D culture models have recently been used to investigate the effects of elevated IFP, interstitial flow, and mechanical compression on the invasive phenotype of breast cancer cells [103, 123, 124, 188, 189]. In one study, a hydrostatic pressure gradient was applied across MDA-MB-231 human breast cancer cells embedded sparsely in an ECM gel [123]. Interstitial flow was found to increase the percentage of cells that migrated as well as their speed, suggesting that IFP can enhance tumor invasion [123]. Cells migrated parallel to the direction of fluid flow via autologous chemotaxis, a phenomenon that was also observed in 3D Boyden chambers [187]. In a similar study, applying a hydrostatic pressure gradient across a single cell suspension of the same cell line in a type I collagen gel induced the cells to migrate primarily against the direction of flow at higher seeding densities [124, 188]. Interstitial flow has also been shown to influence the type of cell motility, promoting ameoboid over mesenchymal migration in MDA-MB-231 cells [255]. It is clear that elevated IFP and interstitial flow influence the migration of cancer cells in culture, though the underlying molecular mechanisms are unclear [176].

The specific responses of cancer cells to IFP appear to depend heavily on experimental context. In many solid breast tumors, cells do not exist in isolation; rather, they are packed together, much as in normal tissues. To recapitulate the aggregate nature of cells in a solid tumor, we recently developed an engineered 3D culture model and used this system to apply a hydrostatic pressure drop across a packed aggregate of MDA-MB-231 human breast
cancer cells. This model yields a more physiologically relevant system to study collective cell behavior, such as IFP-induced invasion [256]. We found that cells formed multicellular chains that invaded collectively from the tips of the aggregates. In addition, we found that invasion occurred against the direction of flow [189]. Surprisingly, interstitial hypertension (i.e. elevated IFP at the base of the aggregates relative to the IFP at the tip) inhibited invasion from the tumor margin. Pressure-induced convection of tumor-conditioned medium controlled the invasive phenotype at the margin of the engineered tumors.

Here we took advantage of this 3D engineered tumor model to define the effects of IFP on invasion-related gene expression in human breast and prostate cancer cells, focusing specifically on genes associated with EMT [37]. Using quantitative RT-PCR, we measured the relative transcript levels of several genes under three pressure profiles: control (i.e. uniform IFP), interstitial hypertension, and interstitial hypotension (elevated IFP at the tip of the aggregates relative to the base). We found that interstitial hypertension inhibited invasion and decreased expression of both mesenchymal and epithelial markers. Conversely, interstitial hypotension promoted invasion in part by increasing expression of the mesenchymal genes vimentin and Snail. Surprisingly, interstitial hypotension also increased the expression of epithelial markers E-cadherin and keratin-8. Using overexpression and RNAi strategies, we found that IFP-induced changes in gene expression were required to produce the observed invasive phenotype. Finally, we found that individual cells in aggregates under non-uniform IFP were more motile than those under uniform IFP and that these characteristics were also modulated by changes in the expression levels of EMT markers. Together, these data highlight the effects of IFP on tumor cell migration and collective invasion in a physiologically relevant 3D culture model, and provide insight into the molecular mechanisms underlying the effects of IFP on invasive phenotype in solid tumors.
5.2 Results

5.2.1 Interstitial fluid pressure regulates collective invasion from tumor cell aggregates

We used a 3D microfluidic culture model to engineer intact aggregates of MDA-MB-231 human breast cancer cells embedded within a gel of type I collagen (Figure 5.1A). Briefly, a needle was used to mold a blind-ended channel within the gel, which was filled with a concentrated suspension of cells [189]. This procedure yielded an aggregate of cells, the base of which mimicked the core of a solid tumor while the tip mimicked the tumor periphery. Tumor aggregates were subjected to different hydrostatic pressure profiles by independently varying the pressure at the base of the aggregate ($P_{\text{base}}$) from that at the tip ($P_{\text{tip}}$). This system allowed us to determine the effects of IFP on collective invasion. Invasions were defined as multicellular protrusions emanating from aggregate tips (Figure 5.1B) as determined using a nuclear stain (Figure 5.1C). The characteristically collective invasions that we observed are consistent with reports by others using the same cell line [4, 46].

Under the control pressure profile ($P_{\text{base}}=P_{\text{tip}}$), 25% of aggregates developed invasions (Figure 5.1D). Under $P_{\text{base}}>P_{\text{tip}}$, which mimics the interstitial hypertension frequently observed in solid tumors, invasions were absent (0 out of 51 samples). In contrast, when aggregates were subjected to interstitial hypotension ($P_{\text{base}}<P_{\text{tip}}$), 50% developed invasions. Even moderate pressure profiles of either type ($P_{\text{base}}\geq P_{\text{tip}}$ or $P_{\text{base}}\leq P_{\text{tip}}$) affected invasion frequency. IFP similarly controlled invasion from aggregates of PC-3 prostate cancer cells (Figure 5.2A). These data suggest that interstitial hypertension prevents invasion, whereas interstitial hypotension promotes invasion. Cells thus invaded collectively primarily against the direction of fluid flow, which is consistent with what others have reported for densely seeded single-cell suspensions of MDA-MB-231 cells [124, 188].
Figure 5.1: IFP determines the invasive phenotype of human breast cancer aggregates. (A) Schematic of the 3D culture model. *Top panel:* perspective view of a control pressure profile. *Middle panel:* side view. *Bottom panel:* perspective view showing the use of a gasket to apply a pressure difference. (B) Phase-contrast image of an aggregate under $P_{\text{base}} < P_{\text{tip}}$. Arrow
indicates collective invasion. Dashed yellow line denotes the shape of the aggregate at day 0. (C) Hoechst 33342 staining of cell nuclei (white). (D) Frequency of invasion of aggregates under $P_{\text{base}}=P_{\text{tip}}$ ($n=49$), $P_{\text{base}}<P_{\text{tip}}$ ($n=50$), or $P_{\text{base}}>P_{\text{tip}}$ ($n=51$). (E) Phase-contrast image of an aggregate under $P_{\text{base}}>P_{\text{tip}}$. Arrow indicates invadopodia. Inset shows magnified image of boxed region. (F) Average length of invadopodia for aggregates under $P_{\text{base}}=P_{\text{tip}}$ ($n=49$), $P_{\text{base}}<P_{\text{tip}}$ ($n=49$), or $P_{\text{base}}>P_{\text{tip}}$ ($n=55$). (G) Invadopodial length density for aggregates under $P_{\text{base}}=P_{\text{tip}}$ ($n=57$), $P_{\text{base}}<P_{\text{tip}}$ ($n=51$), or $P_{\text{base}}>P_{\text{tip}}$ ($n=56$). (H) Frequency of invasion with ($n=26$) and without ($n=27$) GM6001 in aggregates under $P_{\text{base}}<P_{\text{tip}}$. (I) Invadopodial length densities with ($n=26$) and without ($n=25$) GM6001 in samples under $P_{\text{base}}>P_{\text{tip}}$. * $P<0.05$, *** $P<0.001$. Scale bars, 100 $\mu$m.

Although it prevented invasion, interstitial hypertension induced the formation of anuclear membrane protrusions (Figure 5.1E) that resembled invadopodia, membrane protrusions of tumor cells that locally degrade the ECM [257]. We quantified the response of these putative invadopodia to IFP by measuring their average length and density. Aggregates under hypertension displayed longer and denser invadopodia than those under control and hypotension pressure conditions (Figure 5.1F, G). Similar results were observed in samples under moderate hypertension or hypotension pressure profiles ($P_{\text{base}}\geq P_{\text{tip}}$ or $P_{\text{base}}\leq P_{\text{tip}}$) (Figure 5.2B). The density of putative invadopodia did not correlate with the magnitude of the pressure profile for interstitial hypertension (Figure 5.2C).

The formation of invadopodia is believed to precede invasion by tumor cells [258]. We therefore sought to determine whether the membrane protrusions observed in our samples primarily under hypertension displayed characteristic features of invadopodia, such as the ability to degrade the ECM. We treated samples with the broad-spectrum MMP inhibitor, GM6001, which prevented invasion under hypotension (Figure 5.1H), suggesting that matrix remodeling is required for collective invasion. Treatment with GM6001 did not, however, suppress the formation of putative invadopodia under hypertension (Figure 5.1I). We also stained samples for the invadopodial markers cortactin, Tks5, phosphorylated FAK (pY$_{397}$) [259], and MMP14. The multicellular collective invasions that formed under hypotension strongly expressed these markers (Figure 5.3). The putative invadopodia in samples under hypertension were enriched in cortactin and Tks5. Staining for MMP14 was weak, consistent with
Figure 5.2: IFP controls invasive phenotype of breast and prostate cancer cells. (A) Frequency of invasion of PC-3 cell aggregates under $P_{base}=P_{tip}$ ($n=27$), $P_{base}<P_{tip}$ ($n=20$), or $P_{base}>P_{tip}$ ($n=17$). (B) Invadopodial length density for MDA-MB-231 cell aggregates under $P_{base} \leq P_{tip}$ or $P_{base} \geq P_{tip}$. (C) Invadopodial density as a function of pressure profile for MDA-MB-231 breast cancer aggregates. The solid line denotes the best least-squares linear fit. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

the behavior of GM6001-treated samples (Figure 5.3). The levels of phosphorylated FAK in the putative invadopodia were also weak. These results suggest that the anuclear membrane protrusions observed in the tumor aggregates are non-functional invadopodia—even though they express some invadopodial markers, they are unable to degrade the ECM.
Figure 5.3: Confocal images of immunofluorescence stains for cortactin, MMP14, Tks5, and phosphorylated FAK (pY-FAK) in MDA-MB-231 cell aggregates under IFP. Arrows denote invasive protrusions containing multiple nuclei; the inset in the right cortactin image magnifies the dotted area. Scale bars, 100 μm.
5.2.2 Interstitial fluid pressure induces changes in the expression of EMT-associated markers

To determine the molecular mechanisms underlying IFP-induced invasive phenotypes, we performed a targeted gene expression analysis over several days of culture using quantitative RT-PCR. We focused on genes associated with EMT, a phenotypic switch often implicated in tumor cell invasion [4]. Specifically, we examined the expression of the epithelial markers E-cadherin and keratin-8 and the mesenchymal markers vimentin and Snail. The forward and reverse primers used are provided in Table 5.1. The expression of these genes has previously been shown to be modulated by fluid flow in ovarian cancer cells [260]. Moreover, the invasive response of breast cancer cells to interstitial fluid flow has been shown to vary depending on expression levels of EMT markers [261].

Table 5.1: Primers used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>Fwd: CGGCGACGACCCCATTCGAAC  &lt;br&gt; Rev: GAATCGAACCCTGATTTCCCGTC</td>
</tr>
<tr>
<td>VIM</td>
<td>Fwd: ATCAACACCGAGTTCAAG  &lt;br&gt; Rev: GCCAGCAGGATCTTATTC</td>
</tr>
<tr>
<td>SNAI1</td>
<td>Fwd: CCACTCAGATGTCAAGAAG  &lt;br&gt; Rev: GCAGGTATGGAGAGGAAG</td>
</tr>
<tr>
<td>CDH1</td>
<td>Fwd: CTAAATTCTGATTCTGTGCTTTTG  &lt;br&gt; Rev: CCTCTTCTCCGCCTCCTC</td>
</tr>
<tr>
<td>KRT8</td>
<td>Fwd: AGTTACCGGTCAACCAGAG  &lt;br&gt; Rev: GTCTCCAGCATCTTTGTC</td>
</tr>
</tbody>
</table>

We found that under interstitial hypotension, which promotes invasion, the expression of vimentin and Snail were significantly elevated over several days compared to other IFP profiles, with the most striking effects observed on days 5 and 6 (Figure 5.4). The levels of vimentin and Snail in aggregates subjected to hypertension were not significantly different from the control. Surprisingly, we found that even though hypotension promoted invasion, epithelial genes were significantly upregulated in these aggregates (Figure 5.4) and downregulated in those under hypertension. These data suggest that the invasive phenotypes observed
under different pressure profiles might result from changes in the expression of genes associated with EMT. Furthermore, elevated expression of mesenchymal and epithelial markers correlates with invasion in this system.

Figure 5.4: IFP regulates the expression of EMT markers in human breast cancer aggregates. (A)-(D) Relative transcript levels of vimentin (VIM), Snail (SNAI1), E-cadherin (CDH1), and keratin-8 (KRT8) in aggregates under $P_{\text{base}} = P_{\text{tip}}$ ($n = 4$), $P_{\text{base}} < P_{\text{tip}}$ ($n = 4$), or $P_{\text{base}} > P_{\text{tip}}$ ($n = 4$) over 3-6 days of culture, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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5.2.3 Snail promotes collective invasion and controls invasion length

We next investigated whether the above-described changes in gene expression were required to alter collective invasion in response to IFP. We ectopically expressed Snail by transducing MDA-MB-231 cells with a recombinant adenovirus, resulting in a four-fold increase in Snail as determined by quantitative RT-PCR (Figure 5.5A) and immunoblotting analysis (Figure 5.5B). Ectopic expression of Snail increased the frequency of invasion compared to control ($P < 0.0001$), even under hypertension, indicating that Snail is sufficient to induce an invasive phenotype (Figure 5.6A, B). Snail also increased the number ($P = 0.0003$) (Figure 5.6C) and total length ($P < 0.0001$) (Figure 5.6D) of multicellular invasions from individual aggregates compared to control. Invadopodia still formed under hypertension in Snail-overexpressing tumor aggregates (Figure 5.6B, inset); under hypertension, there was no difference in invadopodial density between AdGFP ($1.94 \pm 0.13 \, \mu m/\mu m$) and AdSnail ($2.06 \pm 0.14 \, \mu m/\mu m$) samples.

To determine whether Snail is necessary for invasion in response to IFP, we used an shRNA approach to stably deplete its levels. MDA-MB-231 cells were transfected with an shRNA construct targeting Snail (shSnail). The resulting stable cell line showed 75% knockdown of Snail transcript compared to scrambled shRNA control (shScr; Figure 5.5C) as well as a reduction in Snail protein (Figure 5.5D). Aggregates expressing shSnail under control or hypotension pressure conditions had a slightly reduced frequency of invasion compared to the shScr control though differences were not statistically significant (Figure 5.6E). Similar to shScr controls, shSnail aggregates did not invade under hypertension. Under hypotension, shSnail aggregates produced short multicellular invasions (Figure 5.6F), but invadopodia were still present (Figure 5.6F, inset). We observed no difference in invadopodial density between shScr ($2.00 \pm 0.14 \, \mu m/\mu m$) and shSnail ($1.97 \pm 0.15 \, \mu m/\mu m$) under hypertension, indicating that Snail is not required for invadopodia formation. Furthermore, shSnail did not significantly affect the number of multicellular collective invasions that formed (Figure 5.6G).
but did decrease their total length ($P = 0.0004$) (Figure 5.6H) as compared to control. These results suggest that Snail promotes collective invasion in response to IFP and may play a role in the lengthening of invasions.

Figure 5.5: Snail expression in MDA-MB-231 cells. (A) Relative transcript levels of Snail in AdSnail ($n = 3$) or AdGFP ($n = 4$) MDA-MB-231 cells. (B) Immunoblot analysis of MDA-MB-231 cells transduced with AdGFP or AdSnail. (C) Relative transcript levels of Snail in shScr ($n = 3$) or shSnail ($n = 3$) MDA-MB-231 cells. (D) Immunoblot analysis for Snail in shScr and shSnail MDA-MB-231 cells. * $P<0.05$, ** $P<0.01$.

**5.2.4 Vimentin is required for collective invasion and formation of invadopodia**

We performed a similar series of experiments to examine the role of vimentin in IFP-mediated invasion. Vimentin was expressed ectopically in MDA-MB-231 cells (Figure 5.7A, B), which increased the frequency ($P = 0.0002$), number ($P = 0.0007$), and total lengths ($P < 0.0001$) of multicellular collective invasions compared to control (Figure 5.8A-D). Ectopic expression of vimentin did not significantly affect invadopodial density ($2.17 \pm 0.09 \mu m/\mu m$) compared to vector control ($2.00 \pm 0.12 \mu m/\mu m$) under hypertension.

To determine whether vimentin expression is necessary for invasion in the engineered tumors, we stably decreased its levels using shRNA (shVim; Figure 5.7C, D). Depleting vimentin completely blocked invasion from the shVim aggregates compared to shScr control
Figure 5.6: Snail controls invasion in response to IFP. (A) Frequency of invasion of AdGFP or AdSnail aggregates under $P_{base}=P_{tip}$ (AdGFP: $n=18$; AdSnail: $n=19$), $P_{base}<P_{tip}$ (AdGFP: $n=12$; AdSnail: $n=17$), or $P_{base}>P_{tip}$ (AdGFP: $n=16$; AdSnail: $n=12$). (B) Phase-contrast image of an AdSnail aggregate under $P_{base}>P_{tip}$. Arrow indicates collective invasion. Inset shows magnified image of boxed region. (C) Number and (D) length of invasions in AdGFP or AdSnail aggregates under IFP. (E) Frequency of invasion of shScr or shSnail aggregates under $P_{base}=P_{tip}$ (shScr: $n=23$; shSnail: $n=24$), $P_{base}<P_{tip}$ (shScr: $n=23$; shSnail: $n=25$), or $P_{base}>P_{tip}$ (shScr: $n=9$; shSnail: $n=11$). (F) Phase-contrast image of an shSnail aggregate under $P_{base}<P_{tip}$. Arrows indicate short collective invasion. Inset shows magnified image of boxed region. (G) Number and (H) length of invasions in shScr or shSnail aggregates under IFP. * $P<0.05$, ** $P<0.01$. Scale bars, 100 µm.
\( P = 0.0008 \) (Figure 5.8E) even under hypotension (Figure 5.8F-H). These results suggest that vimentin is required for IFP-induced invasion. In addition to inhibiting collective invasion, shVim also inhibited the formation of invadopodia (Figure 5.8F, inset). Even under hypertension, invadopodial density was significantly decreased \( (P < 0.0001) \) in shVim samples \( (0.40 \pm 0.06 \ \mu m/\mu m) \) compared to shScr \( (2.06 \pm 0.19 \ \mu m/\mu m) \), which is consistent with the prevailing hypothesis that vimentin-containing intermediate filaments are required for the elongation of invadopodia [262].

![Figure 5.7: Vimentin expression in MDA-MB-231 cells. (A) Relative transcript levels of vimentin in vector \((n = 3)\) or vimentin \((n = 3)\) MDA-MB-231 cells. (B) Immunoblot of vector or vimentin MDA-MB-231 cells. (C) Relative transcript levels of vimentin in shScr \((n = 3)\) or shVim \((n = 3)\) MDA-MB-231 cells. (D) Immunoblot analysis for vimentin in shScr or shVim MDA-MB-231 cells. * \( P < 0.05 \), *** \( P < 0.001 \).](image)

5.2.5 E-cadherin promotes extensive collective invasion

Because E-cadherin levels were elevated under hypotension (Figure 5.4), which promoted invasion, we hypothesized that E-cadherin-mediated intercellular adhesion might be important for collective invasion under IFP. We created a stable line of MDA-MB-231 cells that expressed elevated levels of an E-cadherin-GFP fusion protein (Ecad-GFP; Figure 5.9A, B), which surprisingly increased the frequency \( (P < 0.0001) \), number \( (P < 0.0001) \), and total lengths \( (P < 0.0001) \) of invasions compared to control (Figure 5.10A-D), and permitted
Figure 5.8: Vimentin is required for collective invasion in response to IFP. (A) Frequency of invasion of vector or vimentin-expressing aggregates under $P_{\text{base}}=P_{\text{tip}}$ (vector: $n=16$; vimentin: $n=20$), $P_{\text{base}}<P_{\text{tip}}$ (vector: $n=16$; vimentin: $n=16$), or $P_{\text{base}}>P_{\text{tip}}$ (vector: $n=15$; vimentin: $n=14$). (B) Phase-contrast image of a vimentin aggregate under $P_{\text{base}}>P_{\text{tip}}$. Arrow indicates collective invasion. Inset shows magnified image of boxed region. (C) Number and (D) length of invasions in vector or vimentin aggregates under IFP. (E) Frequency of invasion of shScr or shVim aggregates under $P_{\text{base}}=P_{\text{tip}}$ (shScr: $n=16$; shVim: $n=15$), $P_{\text{base}}<P_{\text{tip}}$ (shScr: $n=15$; shVim: $n=15$), and $P_{\text{base}}>P_{\text{tip}}$ (shScr: $n=9$; shVim: $n=9$). (F) Phase-contrast image of an shVim aggregate under $P_{\text{base}}<P_{\text{tip}}$. Inset shows magnified image of boxed region. (G) Number and (H) length of invasions in shScr or shVim aggregates under IFP. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Scale bars, 100 µm.
formation of invadopodia (Figure 5.10B, inset). There was no difference in invadopodial density between Ecad-GFP (2.02 ± 0.11 µm/µm) and YFP (2.04 ± 0.12 µm/µm) aggregates under hypertension. We observed similar trends in Ecad-GFP-expressing PC-3 aggregates (Figure 5.11). E-cadherin thus appears to promote collective invasion under IFP.

To determine whether E-cadherin is required for collective invasion, we stably decreased its levels in the aggregates using shRNA (shEcad; Figure 5.9C). Similar to shSnail, shEcad reduced the frequency of invasion under control or hypotension pressure conditions compared to the shScr control though these differences were not statistically significant (Figure 5.10E). ShEcad aggregates (Figure 5.10F) produced a similar number of invasions per aggregate as shScr (Figure 5.10G), but the total lengths of invasion per aggregate were shorter ($P = 0.0043$) (Figure 5.10H). ShEcad aggregates did not invade under hypertension (Figure 5.10E), and invadopodial density was unaffected (2.09 ± 0.15 µm/µm) compared to shScr controls (2.02 ± 0.11 µm/µm). These results suggest that, like Snail, E-cadherin promotes invasion and may be necessary for extensive collective invasion in response to IFP.

Figure 5.9: E-cadherin expression in MDA-MB-231 cells. (A) Relative transcript levels of E-cadherin in YFP ($n = 3$) or Ecad-GFP ($n = 3$) MDA-MB-231 cells. (B) Immunoblot analysis for E-cadherin in YFP or Ecad-GFP MDA-MB-231 cells. (C) Relative transcript levels of E-cadherin in shScr ($n = 3$) or shEcad ($n = 3$) MDA-MB-231 cells. (D) Immunofluorescence staining for β-catenin (red) and Hoechst 33342 staining of cell nuclei (blue) in invasions from an Ecad-GFP (green) aggregate under $P_{base}<P_{tip}$. * $P<0.05$, ** $P<0.01$. Scale bar, 25 µm.
Confocal fluorescence imaging revealed that E-cadherin was diffuse (Figure 5.12A, B) and co-localized with β-catenin in Ecad-GFP-expressing aggregates (Figure 5.9D). No clear cell-cell junctions were discernible, suggesting that transient intercellular contacts may be sufficient for the extensive invasion observed. To determine whether E-cadherin levels per se increase ability to invade, we created mosaic aggregates that contained 30% YFP or Ecad-GFP cells. As expected, multicellular invasions from YFP-mosaic aggregates contained ∼30% YFP-expressing cells. In contrast, multicellular invasions from Ecad-GFP-mosaic aggregates (under hypotension) contained ∼96% Ecad-GFP-expressing cells (Figure 5.12C). These data suggest that high levels of E-cadherin promote collective invasion in response to IFP and that invasions are enriched in cells with high levels of E-cadherin. These data may help clarify the clinical finding that breast cancer cells in metastases express higher levels of E-cadherin than those in the primary tumor [49] by predicting that E-cadherin-overexpressing cells preferentially form initial invasions from the primary site.

5.2.6 Cells within aggregates are more motile under IFP gradients

IFP regulates the collective invasion of MDA-MB-231 cells from an engineered primary tumor. This finding suggested that IFP might affect the motility of individual cells. We labeled the nuclei of cells in aggregates with H2B-mCherry, and tracked cell positions over 20 hours of culture (Figure 5.13A-C). Under hypotension, invading cells migrated farthest from their initial positions (Figure 5.13B) and against the direction of fluid flow, consistent with recent single-cell suspension studies [124, 188]. We also noted that invading cells migrated in a pulsatile fashion, occasionally moving backwards rather than only progressing forward, congruent with recent observations of collective invasions of normal cells [167]. Cells under hypertension were also more motile than controls (Figure 5.13C) despite the fact that these cells did not invade the surrounding ECM. This suggests that gradients in interstitial fluid pressure enhance cell motility without necessarily resulting in invasion.
Figure 5.10: E-cadherin promotes invasion in response to IFP. (A) Frequency of invasion of YFP or Ecad-GFP-expressing aggregates under $P_{\text{base}}=P_{\text{tip}}$ (YFP: $n=16$; Ecad-GFP: $n=22$), $P_{\text{base}}<P_{\text{tip}}$ (YFP: $n=17$; Ecad-GFP: $n=17$), or $P_{\text{base}}>P_{\text{tip}}$ (YFP: $n=10$; Ecad-GFP: $n=13$). (B) Phase-contrast image of an Ecad-GFP aggregate under $P_{\text{base}}<P_{\text{tip}}$. Arrows indicate collective invasions. Inset shows magnified image of boxed region. (C) Number of invasions and (D) length of invasions in YFP or Ecad-GFP aggregates under IFP. (E) Frequency of invasion of shScr or shEcad aggregates under $P_{\text{base}}=P_{\text{tip}}$ (shScr: $n=13$; shEcad: $n=14$), $P_{\text{base}}<P_{\text{tip}}$ (shScr: $n=10$; shEcad: $n=15$), or $P_{\text{base}}>P_{\text{tip}}$ (shScr: $n=9$; shEcad: $n=9$). (F) Phase-contrast image of a shEcad aggregate under $P_{\text{base}}<P_{\text{tip}}$. Arrow indicates collective invasion. Inset shows magnified image of boxed region. (G) Number and (H) length of invasions in
shScr or shEcad aggregates under IFP. * P<0.05, ** P<0.01, *** P<0.001. Scale bars, 100 µm.

Figure 5.11: Ectopic expression of E-cadherin promotes invasion, whereas depletion of E-cadherin inhibits extensive invasion in response to IFP in PC-3 cells. (A) Frequency of invasion of YFP or Ecad-GFP-expressing PC-3 aggregates under $P_{\text{base}}=P_{\text{tip}}$ (YFP: $n=36$; Ecad-GFP: $n=25$), $P_{\text{base}}<P_{\text{tip}}$ (YFP: $n=16$; Ecad-GFP: $n=19$), or $P_{\text{base}}>P_{\text{tip}}$ (YFP: $n=15$; Ecad-GFP: $n=17$). (B) Number and (C) length of invasions in YFP or Ecad-GFP PC-3 aggregates under IFP. (D) Relative transcript levels of E-cadherin in YFP ($n=3$) or Ecad-GFP ($n=3$) PC-3 cells. (E) Immunoblot analysis for E-cadherin in YFP- or Ecad-GFP-transfected PC-3 cells. * P<0.05, ** P<0.01.
Figure 5.12: Cells expressing ectopic E-cadherin preferentially undergo collective invasion. (A) Phase-contrast image of an invasive protrusion from an Ecad-GFP aggregate under $P_{base} < P_{tip}$. (B) Confocal image of the invasion from (A) showing Ecad-GFP localization (green) and Hoechst 33342 staining of cell nuclei (blue). (C) Percentage of fluorescent cells in invasions in aggregates under $P_{base} < P_{tip}$ from YFP or Ecad-GFP mosaic aggregates. *** $P<0.001$. Scale bars, 50 µm.

Non-invading cell tracks were used to compute net displacement, path length, and cell speed. Cells in aggregates under hypotension or hypertension pressure conditions migrated further from their starting positions and had longer path lengths than cells under the control pressure condition (Figure 5.13D-F). The directionality ratio, a measure of persistence, was higher for cells under hypotension (0.32 ± 0.04) and hypertension (0.27 ± 0.03) compared to the control pressure condition (0.20 ± 0.03). Cells under IFP gradients also migrated faster; cells under the control pressure condition had an average speed of 3.35 ± 0.21 µm/hr compared to hypotension (5.80 ± 0.31 µm/hr) and hypertension (5.47 ± 0.51 µm/hr). Expression
of Ecad-GFP increased net displacement as well as path length (Figure 5.13G). Ecad-GFP cells also migrated faster (7.43 ± 0.45 µm/hr) than YFP control cells (6.17 ± 0.60 µm/hr). We also noted a subset of non-invading cells within the Ecad-GFP aggregates that migrated together in small groups (2-4 cells) (Figure 5.13H). These data suggest that IFP controls collective invasion by modulating the motility of individual cells, in part through E-cadherin.

5.3 Discussion

Our data provide molecular insight into how IFP, a physical property of the tumor microenvironment, modulates the collective behavior of tumor cells. In our previous study, we found that the direction of IFP-induced flow regulated the invasive phenotype by altering the chemical composition of the interstitial fluid near the surface of the aggregates [189]. Here, we further probed the effects of IFP on invasion by focusing on gene expression and found that the direction of IFP gradients alters the expression levels of the EMT-associated markers Snail, vimentin, and E-cadherin. Elevated expression of these markers promotes collective invasion, for which vimentin is absolutely required. In addition to affecting the invasive phenotype of multicellular aggregates, IFP also affects the persistence and motility of individual cells, which are regulated by EMT-marker expression. Our system provides insights into the results of single-cell suspension culture models and illuminates how IFP affects collective cell behavior [123, 124, 188, 255].

The predominant form of invasion from solid tumors is collective in nature and histological sections of many epithelial tumors reveal collective invasion [7, 222, 223]. Our data suggest that cells need not necessarily exhibit a completely mesenchymal phenotype in order to invade collectively from a solid tumor. We observed that aggregates with the highest frequency of collective invasion had elevated levels of both mesenchymal and epithelial markers and that E-cadherin in particular promoted collective invasion, which suggests an additional role for EMT markers in the cellular response to physical cues such as IFP. These results are
Figure 5.13: Cells in aggregates under elevated IFP are motile and persistent. Data are representative of cells in aggregates from three independent experiments. Overlaid individual cell tracks from aggregates under (A) $P_{\text{base}}=P_{\text{tip}}$ ($n=16$), (B) $P_{\text{base}}<P_{\text{tip}}$ ($n=18$), or (C) $P_{\text{base}}>P_{\text{tip}}$ ($n=15$) are shown. Arrows indicate direction of fluid flow. Light grey tracks represent cells undergoing collective invasion. Net displacement (black lines) and path length (grey lines) for non-invading cells in aggregates under (D) $P_{\text{base}}=P_{\text{tip}}$, (E) $P_{\text{base}}<P_{\text{tip}}$, and (F) $P_{\text{base}}>P_{\text{tip}}$. (G) Net displacement (black lines) and path length (grey lines) for YFP (solid lines; $n=15$) or Ecad-GFP (dotted lines; $n=21$) cells in aggregates under $P_{\text{base}}<P_{\text{tip}}$. (H) Overlaid individual cell tracks from non-invading Ecad-GFP cells under $P_{\text{base}}<P_{\text{tip}}$ ($n=8$). Light grey tracks indicate cells moving together as a cohort.
consistent with those from other studies showing that E-cadherin expression may actually enhance metastatic potential [49, 263, 264], which contrasts with the classical view of E-cadherin as a tumor suppressor [157, 265, 266, 267, 268]. Remarkably, we found that E-cadherin was enriched in invading cohorts. While it is perhaps counterintuitive for a cell-cell adhesion protein to promote invasion, E-cadherin might permit the directed motion of cohorts by maintaining cohesion between cells [4]. Cell-cell contacts could facilitate the long-range transmission of force, guiding cells in the rear of a cohort to migrate in the same direction as those at the leading edge. We observed this phenomenon in timelapse imaging of Ecad-GFP aggregates; cohorts of cells migrating together had highly correlated motion. Consistently, disrupting adherens junctions was found to impair the directional motion of collectively migrating epithelial cells [269]. Interestingly, however, the localization of E-cadherin in E-cad-GFP invasions was not purely junctional, and E-cadherin appeared to also be present in the cytoplasm, suggesting that aberrant E-cadherin expression could also be contributing to increased invasiveness in our system, consistent with previous studies in human breast cancer samples [49, 270]. These data support the theory that tumor cells do not need to transition entirely to a mesenchymal cell fate in order to invade from a primary tumor.

In response to IFP, changes in gene expression preceded changes in phenotype as differences in EMT marker levels persisted over several days prior to invasion. Maintained expression of these proteins may therefore be required to induce downstream signaling for collective invasion in response to IFP. Snail and vimentin are known to interact with key mechanotransduction pathway components: Snail can activate myosin II [271], and vimentin can activate ERK and protect it from dephosphorylation, in addition to binding to protein kinase B (PKB) to escape proteolysis [272, 273, 274, 275]. Vimentin also plays a role in conferring mechanical stability, protecting cells against compressive stress as well as enhancing their elastic behavior and contractility [276], which may in turn mediate invasion. In endothelial cells, fluid flow results in rapid translocation of vimentin to the nucleus and near cell-cell junctions [277], indicating that vimentin is part of the cellular response to physical
signals, translating deformations at the cell surface to an intracellular reaction. Consistently, depleting vimentin completely inhibited invasion under all IFP profiles in our system, suggesting that these cells could not respond to IFP. E-cadherin is also involved in the cellular response to mechanical cues via transcription regulation [278]. The amplified invasive phenotype of Ecad-GFP aggregates could be related to an enhanced response to IFP. Our data suggest that these EMT markers are required for the translation of physical cues such as IFP into changes in cell behavior.

IFP is known to affect the transport and delivery of cancer therapeutics [182]. Importantly, our results suggest that elevated IFP in vivo, which manifests as interstitial hypertension, also affects cancer cell behavior. Interstitial hypertension may in fact inhibit invasion from the boundary of a primary tumor into the surrounding tissue space. As such, commonly prescribed therapeutics that directly affect IFP may also affect the invasive phenotype of cells in a tumor. Our results also suggest that, within a mechanically heterogeneous tumor, local hypotension could promote upstream cell migration. The main source of interstitial fluid in tumors is the leaky vasculature [183], therefore tumor cells within close proximity to intratumoral blood vessels, where the local vascular pressure is relatively higher than the interstitial fluid pressure, may be prone to migrate upstream towards these vessels and potentially metastasize [124]. Determining how IFP contributes to tumor phenotype will be critical for a complete understanding of the interaction between tumor cells and their local microenvironment.

5.4 Materials and methods

5.4.1 3D engineered tumor model

MDA-MB-231 human breast cancer cells and PC-3 human prostate cancer cells (passed at a 1:4 ratio, and used from passage 6-9) were obtained from the American Type Culture Collection and cultured in DMEM/F12 growth medium (Hyclone) or RPMI 1640 growth medium.
medium (Hyclone) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 50 µg/mL gentamicin (Gibco), respectively. Engineered tumors consisted of aggregates of MDA-MB-231 or PC-3 cells embedded in collagen gels housed within chambers of PDMS and were fabricated as described previously [189]. Acid-extracted bovine type I collagen (Koken) was neutralized with NaOH to a pH of 8.5-9 and diluted with cell culture medium to a final concentration of 4 mg/mL. Seeded channels were incubated at 37°C for two days. To apply a hydrostatic pressure gradient, the heights of medium in the reservoirs adjacent to the channel either on the side of the tumor base (P _base_) or on the side of the tumor tip (P _tip_) were altered using PDMS gaskets two days after seeding. The pressure differential |P _base_ - P _tip_| was ∼1.6 cm H2O or ∼1.2 mm Hg. Upon establishment of a pressure differential, flow speed in the channels was calculated to be ∼1 µm/s, similar to what has been reported in vivo [279]. To establish moderate pressure profiles of either type, P _base_ ≥ P _tip_ or P _base_ ≤ P _tip_, we added a slight excess of medium to one side such that |P _base_ - P _tip_| was ∼0.2 cm H2O or ∼0.15 mm Hg. To maintain the pressure profiles, we replenished the medium twice per day. Samples were maintained in culture for up to nine days. To inhibit matrix degradation, GM6001 (40 µM; Calbiochem) was added to the high-pressure reservoir.

5.4.2 Plasmids, transfection, and adenoviral transduction

The cDNA for human vimentin was PCR-amplified from pENTR-vimentin and ligated into the p3xFlag-CMV vector (Sigma Aldrich) using EcoRI and BamHI restriction sites. Empty p3xFlag-CMV vector was used as a control. Expression plasmids for shRNA against Snail, vimentin, and E-cadherin, pLKO.1-Snail-shRNA (shSnail), pLKO.1-vimentin-shRNA (shVim), and pLKO.1-E-cadherin-shRNA (shEcad) were obtained from Sigma Aldrich (TRCN0000063819, TRCN0000029119, and TRCN0000237840, respectively). The control scrambled shRNA plasmid, pLKO.1-scramble-shRNA, was obtained from Addgene (plasmid 1864). Ectopic expression of E-cadherin was achieved using pcDNA3.1-E-cadherin-GFP (Addgene; plasmid 28009) with pcDNA3.1-YFP as a control. MDA-MB-231 or PC-3
cells were transiently transfected with mammalian or shRNA expression constructs using Lipofectamine 3000 transfection reagent (Invitrogen). MDA-MB-231 cells stably expressing E-cadherin, shScr, shSnail, shVim, or shEcad were collected from pooled populations that were selected using G418 (1 mg/mL) or puromycin (1 µg/mL) for three weeks. Ectopic Snail expression was achieved by transducing cells with an adenoviral vector, AdSnail-GFP [143], with AdGFP as a control. For live imaging, cells were transduced with AdH2B-mCherry. Cells were transduced at an MOI resulting in >99% transduction efficiency. For mosaic experiments, aggregates were formed using 30% MDA-MB-231 cells stably expressing YFP or Ecad-GFP and 70% untransduced cells.

5.4.3 Quantitative real-time PCR

Samples were treated with a 2 mg/mL solution of collagenase A (Sigma Aldrich) in cell culture medium for five minutes prior to isolation of total RNA using the Qiagen RNeasy Mini Kit (Qiagen, Inc.). Isolated RNA was used to synthesize cDNA using a Verso cDNA Synthesis Kit (Thermo Scientific). Transcript levels were measured by quantitative real-time PCR using a BioRad Mini-Opticon instrument and the iTaq Universal SYBR Green Supermix (BioRad). Primers for E-cadherin, keratin-8, vimentin, Snail, and 18S rRNA (Supplemental Table 1) were designed using Beacon Designer software (BioRad) and determined to be specific using BLAST and dissociation curve analysis. All transcript levels were normalized to that of 18S rRNA in the same sample. Experiments were performed independently in triplicate, with six samples per treatment condition per experiment.

5.4.4 Timelapse microscopy

Samples transduced with AdH2B-mCherry were imaged beginning seven days after seeding. Timelapse movies were collected using a Hamamatsu C4742-95 camera attached to a Nikon Ti-U inverted microscope and fitted with a humidified environmental chamber held at 37°C
and 5% CO₂. Image stacks (960 µm × 730 µm × 200 µm, spaced 20 µm in the z-direction) were acquired using a Plan Fluor 10×/0.3 NA objective every hour for a total of twenty hours.

5.4.5 Quantitative image analysis

Samples were imaged under phase-contrast microscopy using a 10×/0.4 NA objective on a Nikon Ti-U inverted microscope (Nikon) with a Hamamatsu Orca CCD camera. On days 7-9 after seeding, samples under all pressure conditions were fixed with 4% paraformaldehyde in PBS (added to the well on the higher pressure side for hypertension and hypotension \( P_{\text{base}} \neq P_{\text{tip}} \) or to the well on the \( P_{\text{base}} \) side for the control pressure condition \( P_{\text{base}} = P_{\text{tip}} \)) and washed three times with PBS. Nuclei were labeled using Hoechst 33342 and visualized under UV illumination. Invasions were defined as multicellular protrusions from the aggregate tips. The lengths of these protrusions and invadopodia (anuclear protrusions) were measured using ImageJ software (NIH). Invadopodial density per aggregate was calculated as total length of invadopodia divided by the perimeter of the aggregate. For analysis of live imaging data, cells within 150 µm of aggregate tips were tracked manually using the MTrackJ plugin in Image J. Cell tracks were exported to Microsoft Excel in order to calculate speed, net displacement, path length, and directionality ratio (net displacement divided by path length of a trajectory) using open-source macros from DiPer [280]. Correlations between individual cell tracks were determined using the sample cross-covariance function as described previously [167].

5.4.6 Immunofluorescence analysis

Fixed samples were removed from the PDMS chamber, blocked with 10% goat serum and 0.1% Triton X-100 and incubated overnight in primary antibody diluted 1:200 in blocking buffer. Blocked samples were washed with 0.1% Triton X-100 and incubated overnight with Alexa Fluor-conjugated secondary antibody (Invitrogen) diluted 1:1000 in blocking buffer. After additional washing, samples were visualized using a spinning disk confocal
(BD Biosciences) attached to a Nikon Ti-U microscope using a Plan Fluor 20×/0.45 NA objective. Primary antibodies were used to detect cortactin (Millipore), Tks5 (M-300; Santa Cruz), phosphorylated FAK (on Y397; Invitrogen), MMP14 (Millipore), and β-catenin (Sigma Aldrich). Isotype control staining was negative.

### 5.4.7 Immunoblotting

Samples were lysed with RIPA lysis buffer (Thermo Scientific) supplemented with protease inhibitors (Roche), and protein concentrations were measured using the Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific). Samples were then mixed with Laemmli sample buffer, boiled at 95°C for 5 min, resolved by SDS-PAGE, and transferred to nitrocellulose membranes which were blocked in 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) buffer. Blocked membranes were incubated overnight at 4°C in blocking buffer containing antibodies specific for vimentin (clone VIM-13.2; Sigma Aldrich), Snail (clone L70G2; Cell Signaling), E-cadherin (clone 24E10; Cell Signaling), or β-actin (Cell Signaling). Signals were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare).

### 5.4.8 Statistical analysis

Results were analyzed in GraphPad Prism (GraphPad Software). To compare invasion frequencies, we used Fisher’s exact test. To compare invadopodial lengths or invadopodial length densities, we used the Mann-Whitney U test. To correlate invadopodial length density with hydrostatic pressure differential, we used Spearman’s rank correlation test. To compare relative transcript levels among different pressure conditions, we used ANOVA with Bonferroni’s post-test for multiple comparisons. We used the Student’s t-test to compare relative transcript levels of transduced cells, percentages of YFP- or GFP-expressing cells forming invasions in mosaic experiments, and sample cross-correlation coefficients. To compare the numbers or total lengths of multicellular invasions for gene manipulation conditions, we used
the Chi-Square test and Kruskal-Wallis test with Dunn’s multiple comparison post-test, respectively. Mean values represent at least three independent experiments, and error bars represent standard error of the mean. For all statistical tests, $P < 0.05$ was considered to be statistically significant.
As described in Chapter 1, collective migration is an important phenomenon driving larger-scale movement in a variety of biological contexts. However, as cells migrate they must navigate their complex microenvironment, interpret biochemical and physical information, and adjust their behavior accordingly. We specifically sought to probe the effects of intrinsic (cell-derived) and extrinsic (present in the microenvironment) physical forces on collective cell migration in both developmental and disease contexts with the hypothesis that these signals are key regulators of collective movement. To that end we studied two collective migration processes—mammary gland branching in development and cancer cell invasion.

In order to do this, we developed two engineered 3D culture models to recapitulate the \textit{in vivo} environment and enable control and manipulation of mechanical parameters. The first culture model enabled the creation of arrays of identical 3D epithelial tissues of defined geometry completely embedded within an ECM gel. This method is highly customizable and can be used to create tissues of any desired shape. By controlling the shape of the tissues, one can also control the mechanical stress experienced by cells in different regions. The identical nature of the cultured tissues guarantees high statistical confidence in experimental results. This model was used to determine the physical mechanisms of collective migration and the dynamics of ECM alignment prior to migration. The second culture model was designed to
control the fluid pressure and flow across an aggregate of epithelial cells designed to mimic a solid tissue. This model was used to determine the effects of the IFP profile on the collective invasion of cancer cells. Both models can be used with a variety of different cell lines to study other collective migration processes.

Using the arrayed engineered tissue model, we had previously shown that cells preferentially undergo collective migration in response to mechanical stress [166]. Using TFM, we determined that cells undergo collective migration by exerting a tensile force on their surrounding ECM. A similar phenomenon occurs in other cell types and culture models that we tested. In terms of temporal dynamics, we found that the motion of collectively migrating cohorts is pulsatile, exhibiting both extension and retraction over time. Moreover, these cell cohort dynamics correlated with ECM deformations. Key mechanotransduction players and cellular contractility were found to be the driving force for collective migration. Lastly, we found that prior to migrating, cells in the engineered tissues aligned ECM fibers to create directional microtracks to migrate along.

We used the same model to further investigate the dynamics of cell-induced ECM fiber alignment and determined that alignment occurs on a much shorter timescale than migration. Similar to migration, contractility also plays a vital role in the alignment process; Rho-mediated cytoskeletal tension imposes a strain on the matrix and aligns the pericellular fiber network. Accordingly, manipulating contractility resulted in variations in the characteristic time scale for matrix alignment. While MMP activity is required for migration, we found that it is not crucial for matrix alignment, indicating that cell-induced alignment is primarily a physical mechanism. Lastly, we found that the presence of cell-cell adhesions resulted in a shorter fiber alignment time, likely due to the propagation of cytoskeletal tension across multiple cells in a multicellular tissue.

It is clear that mechano-biological signaling and interactions between cells and their microenvironment are important for collective migration in normal tissues and that similar mechanisms seem to be at play for cancerous ones. However, cancer cells experience several
unique changes in their physical microenvironment that do not occur in normal tissues. Hypothesizing that these differences are likely to influence collective invasion, we sought to examine the effects of elevated IFP, a major physical change experienced by cells within solid tumors, on the invasive phenotype. We used the 3D culture model with fluid pressure control to determine how various IFP profiles influence collective invasion from an aggregate of breast cancer cells. We found that IFP affects the motility and invasive behavior of cancer cells by regulating the expression of EMT genes and that expression levels of these markers are required to drive invasive behavior in response to IFP. We observed similar results in prostate cancer cells, suggesting that these phenomena are relevant to a variety of solid tumors of different origins.

Overall, this dissertation aims to contribute to our knowledge of how and under what conditions cells undergo collective migration in a 3D context. Understanding this fundamental process will provide insight into normal development, regeneration, and disease. Key findings are that cells require mechanical sensing in order to migrate collectively and that physical factors in their microenvironment can greatly influence this process, supporting the idea that biomechanics play a vital role in both development and disease processes.

There are several open questions to pursue in terms of future work. It would be interesting to perform a dynamic TFM analysis using both culture models to observe spatiotemporal changes in cellular forces during the collective migration processes, especially since they are quite dynamic. Moreover, it would be useful to quantify the forces required for matrix alignment as compared to migration, as this may provide insight as to why the timescales for these processes are different. It would also be interesting to determine whether other signaling pathways that affect contractility, such as calcium channels and myosin light chain kinase (MLCK), are required for alignment and migration. In terms of the role of IFP in collective cancer invasion, more in-depth molecular analysis is required to determine the mechanisms by which IFP is able to alter the transcript levels of genes associated with EMT. RNA sequencing would be a useful tool to look at gene clusters that are altered under
different pressure profiles. Lastly, it would also be useful to determine how IFP affects the behavior of other cell types in the heterogeneous tumor microenvironment, such as immune cells and fibroblasts.
Appendix A

Engineering 3D Epithelial Tissues Embedded within ECM

This appendix is adapted from the following publication:


A.1 Materials

Table A.1: Materials required for creating 3D epithelial tissues embedded in type I collagen.

<table>
<thead>
<tr>
<th>Name of Reagent/Equipment</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Comments/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polydimethylsiloxane (PDMS)</td>
<td>Ellsworth Adhesives</td>
<td>Sylgard 184</td>
<td></td>
</tr>
<tr>
<td>PDMS curing agent</td>
<td>Ellsworth Adhesives</td>
<td>Sylgard 184</td>
<td></td>
</tr>
<tr>
<td>Lithographically patterned silicon master</td>
<td>self-made</td>
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<td></td>
</tr>
<tr>
<td>Plastic weigh boat</td>
<td>Fisher Scientific</td>
<td>08-732-115</td>
<td></td>
</tr>
<tr>
<td>100-mm-diameter Petri dishes</td>
<td>BioExpress</td>
<td>D-2550-2</td>
<td></td>
</tr>
<tr>
<td>Item</td>
<td>Supplier</td>
<td>Code</td>
<td>Notes</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------------------------------</td>
<td>-----------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ethyl Alcohol 200 Proof</td>
<td>Pharmco-Aaper</td>
<td>111000200</td>
<td>Make a 70% EtOH (v:v) solution by mixing with dH₂O</td>
</tr>
<tr>
<td>Razor blade</td>
<td>American Safety Razor</td>
<td>620179</td>
<td></td>
</tr>
<tr>
<td>1:1 Dulbecco’s Modified Eagle’s Medium : Ham’s F12 Nutrient Mixture</td>
<td>Hyclone</td>
<td>SH30023FS</td>
<td></td>
</tr>
<tr>
<td>(DMEM/F12) (1:1)</td>
<td>Safety Razor</td>
<td>1:1 Dulbecco's</td>
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</tr>
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<td>Fetal Bovine Serum (FBS)</td>
<td>Atlanta Biologicals</td>
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</tr>
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<td>10x Hank’s balanced salt solution (HBSS)</td>
<td>Life Technologies</td>
<td>14185-052</td>
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<tr>
<td>Insulin</td>
<td>Sigma Aldrich</td>
<td>I6634-500MG</td>
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<tr>
<td>Gentamicin</td>
<td>Life Technologies</td>
<td>15750-060</td>
<td></td>
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<tr>
<td>10X Phosphate-buffered saline (PBS)</td>
<td>Fisher Scientific</td>
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<td>Sodium hydroxide (NaOH)</td>
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<tr>
<td>Bovine type I collagen (non-pepsinized)</td>
<td>Koken</td>
<td>IAC-50</td>
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<tr>
<td>Albumin from bovine serum (BSA)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Curved stainless steel tweezers</td>
<td>Dumont</td>
<td>7</td>
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<tr>
<td>35-mm-diameter tissue culture dishes</td>
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<td>15 mL conical tubes</td>
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<td>1.5 mL Eppendorf Safe-Lock Tube</td>
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<tr>
<td>Circular #1 glass coverslips, 15-mm in diameter</td>
<td>Bellco Glass Inc.</td>
<td>Special order</td>
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<td>0.05% 1X Trypsin-EDTA</td>
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<td>Paraformaldehyde</td>
<td>VWR</td>
<td>100503-916</td>
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<td>Triton X-100</td>
<td>Perkin Elmer</td>
<td>N9300260</td>
<td>Detergent</td>
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<tr>
<td>HGF</td>
<td>Sigma Aldrich</td>
<td>H 9661</td>
<td>Resuspended in dH₂O at 50 µg/mL</td>
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<td>Rabbit anti-mouse FAK antibody</td>
<td>Life Technologies</td>
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</tr>
<tr>
<td>Goat anti-rabbit Alexa 488 antibody</td>
<td>Life Technologies</td>
<td>A-11034</td>
<td>Used for color-coding pixel frequency maps.</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------------</td>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>Adobe Photoshop</td>
<td>Adobe</td>
<td>N/A</td>
<td>Free image analysis software used for thresholding, registering, and overlaying images to create a pixel frequency map. The StackReg plugin was used for registering binary images.</td>
</tr>
<tr>
<td>FIJI (ImageJ)</td>
<td>NIH</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

### A.2 Methods

#### A.2.1 Preparation of solutions

1. To prepare a 5 mg/mL solution of insulin, dilute the powdered insulin stock with 5 mM hydrochloric acid (HCl) in dH₂O (500 mg insulin in 100 mL solvent). Prepare 100 mL solvent by adding 50 µL of concentrated HCl to 100 mL distilled water (dH₂O).

2. To make a 1X solution of PBS, dilute the 10X phosphate-buffered saline (PBS) stock solution to 1X with dH₂O under sterile conditions.

3. Prepare the PDMS elastomer solution as follows: mix the PDMS prepolymer together with the curing agent in a 10:1 (w:w) ratio.

4. Prepare the cell culture medium as follows: to 500 mL of stock Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), add 10 mL of sterile fetal bovine serum (FBS), 500 µL of gentamicin reagent, and 500 µL of 5 mg/mL insulin.
5. To prepare the 1% bovine serum albumin (BSA) in 1X PBS solution, add 1% (w:v) solution of BSA powder to 1X PBS and mix thoroughly.

6. To prepare a 4 mg/mL solution of neutralized bovine type I collagen, add 50 µL 10X Hank’s balanced salt solution (HBSS) buffer, 30 µL 0.1 N sodium hydroxide (NaOH), 30 µL cell culture medium, and 400 µL of stock collagen to a chilled 1.5 mL micro-centrifuge tube. Mix slowly by pipeting up and down; avoid introducing bubbles. To remove any bubbles, briefly centrifuge the mixture at 4°C.

7. Prepare a fixative solution by diluting 16% paraformaldehyde 1:4 (v:v) in 1X PBS.

8. Prepare a nuclei labeling solution by diluting the stock nuclei labeling solution 1:1000 (v:v) in 1X PBS.

9. Prepare a 0.3% phosphate-buffered saline and detergent (PBST) solution by mixing 1X PBS with 0.3% (v:v) detergent.

10. Prepare blocking buffer by diluting goat serum 1:10 (v:v) in 0.3% PBST.

11. Prepare a primary antibody solution for a particular protein of interest by diluting primary antibody (for example, rabbit anti-focal adhesion kinase (FAK)) 1:200 (v:v) in blocking buffer.

12. Prepare a secondary antibody solution by diluting a fluorescent probe-conjugated antibody (for example, goat anti-rabbit) 1:1000 (v:v) in blocking buffer.

A.2.2 Preparation of elastomeric stamps for 3D micropatterning

Note: Elastomeric stamps are made with PDMS.

1. Make a PDMS solution at the same ratio as described in Step 1.3 and mix thoroughly. Place the mixture in a vacuum chamber for 15-30 minutes to remove any air bubbles that were introduced during the mixing process.
2. Pour the degassed solution into a plastic weigh boat or Petri dish containing a silicon master that is lithographically patterned with features of desired geometry. For best results, cure the PDMS solution at 60°C for ~12 hours. The silicon masters are highly customizable; this protocol uses rectangular structures with dimensions of 50 µm × 200 µm × 50 µm spaced 200 µm apart. The silicon masters can be made using standard photolithography techniques. Briefly, an epoxy-based photoresist is spin-coated on top of a silicon wafer. A mask detailing the desired stamp features is placed on top of the photoresist-coated wafer, which is then exposed to UV light. The desired features not blocked by the mask are exposed to the light and the photoresist at these locations becomes crosslinked, while the remainder of the photoresist coating is soluble and can be washed away.

3. After the PDMS has cured around the desired features on the silicon master, remove the PDMS and master from the plastic container. Carefully separate the PDMS from the silicon wafer and remove excess PDMS from around the imprinted features using a clean razor blade.

4. Now cut the PDMS patterned with imprinted features into individual rectangular stamps (~8-mm × 5-mm) with a razor blade. Store these feature-side-up in a clean 100-mm-diameter Petri dish.

5. Additionally, use the PDMS solution described previously to make supports for the rectangular stamps.

6. Using a spin coater, spread ~2-3g of the PDMS solution evenly on a 100-mm-diameter Petri dish and cure the PDMS for ~12 hours at 60°C. Then, using a razor blade, cut the thin layer of PDMS into rectangles (~5 mm × 2 mm). Note: Two supports are needed for each PDMS stamp made in Step 2.3. The supports can be stored in the 100-mm-diameter Petri dish that contains the PDMS stamps.
7. Before the PDMS stamps and supports can be used for cell culture, sterilize by immersion in 70% ethanol and dry using an aspirator in a biosafety cabinet (cell culture hood).

A.2.3 Preparation of 3D epithelial tissues

1. In a biosafety cabinet, add a drop of approximately 50 µL of 1% BSA in PBS to the top of each stamp. Place the droplet covered stamps at 4°C for a minimum of 4 h to ensure that the BSA adsorbs to the surface of the stamp.

2. Aspirate BSA solution from the PDMS stamps.

3. Wash the surfaces of the stamps twice with cell culture medium (50 µL per wash per stamp should be sufficient), aspirating after each wash.

4. Handle the sterilized PDMS stamps and supports using curved stainless steel tweezers. In a biosafety cabinet, lay out one 35-mm-diameter tissue culture dish for each PDMS stamp. In each dish, lay down two PDMS supports separated by a distance slightly less than the length of the PDMS stamps.

5. Using the tweezers, sterilize as many circular glass coverslips (15 mm in diameter, #1) as there are PDMS stamps using 70% ethanol. Aspirate the excess liquid from the coverslips while holding them with the tweezers. Store the washed coverslips in a separate 100-mm-diameter Petri dish.

6. Dispense ~50 µL of the collagen mixture to evenly coat the surface of each PDMS stamp.

7. Pick up each collagen-coated PDMS stamp with the tweezers and gently invert them. Lower the inverted stamps on top of the PDMS supports laid out in 35-mm-diameter tissue culture dished such that the collagen is between the stamp and the bottom of the tissue culture dish. Incubate the dishes at 37°C for 30 minutes.
8. Dispense ∼50 µL of the remaining collagen mixture onto each of the circular coverslips (later, these will be placed on top of the engineered tissues to completely encapsulate them in collagen) and incubate them at 37°C for 30 minutes.

9. Obtain a 100-mm-diameter tissue culture dish containing epithelial cells (e.g. EpH4 mouse mammary epithelial cells) at ∼40% confluency. Aspirate the cell culture medium and wash once with 10 mL 1X PBS. Add 2 mL of trypsin to the cells and incubate at 37°C for 5-10 minutes.

10. Add 8 mL of fresh culture medium to the tissue culture dish containing trypsinized cells, detaching any remaining adherent cells from the dish. Mix gently by pipetting and move the cell mixture to a 15 mL conical tube and centrifuge at 100× g for 5 minutes.

11. Aspirate the supernatant from the conical tube and resuspend the cell pellet in cell culture medium. Using a hemocytometer, count the cells to determine the concentration of the suspension. Adjust the suspension volume to obtain a final concentration of ∼10^6–10^7 cells/mL.

12. Remove the gelled collagen samples from the incubator. Using the tweezers, gently lift the PDMS stamps straight upwards to detach them from the molded collagen and discard them.

13. Dispense ∼30 µL of the concentrated cell suspension onto the surface of each collagen gel containing molded cavities of desired geometry. Observe the cells under a brightfield microscope with a 10×/0.25 NA objective while gently shaking the dishes side to side to promote cell settling within the cavities. The cavities should be filled within ∼5 minutes.

14. To remove excess cells from around the cavities, tilt each tissue culture dish on its side and gently dispense ∼400 µL cell culture medium over the surface of the collagen
1. Aspirate the liquid and repeat the wash 1-2 more times, checking the collagen gels under the microscope in between each wash.

15. After the excess cells have been cleared from around the cell-filled cavities in the collagen mold, place the tissue culture dishes into a cell culture incubator at 37°C for 15 minutes. Then, using the tweezers, gently invert the collagen-coated class coverslips and place them on top of the cell-filled collagen molds such that the collagen from the coverslips forms a cap over the cell-filled cavities. Incubate the samples at 37°C for 15 minutes.

16. Once the collagen caps have adhered to the cell-filled collagen mold, dispense ~2-2.5 mL cell culture medium slowly over the glass coverslip on top of the gels. Culture the samples at 37°C for 1-3 days. Note: 24 hours after initial seeding, the epithelial tissues can be treated with growth factors such as EGF or HGF to induce branching.

A.2.4 Immunofluorescence and image analysis

1. Aspirate the cell culture medium from the tissue culture dishes and add enough fixative solution to cover the cell-containing gels. Incubate the dishes at room temperature for 15 minutes on a shaker at 200 rpm.

2. Aspirate the fixative, fill the tissue culture dishes with 1X PBS, and incubate at room temperature for 15 minutes on a shaker at 200 rpm. Repeat twice for three total washes.

3. To label nuclei: Aspirate the PBS from the tissue culture dish and replace with Hoechst solution. Incubate at room temperature for 15-20 minutes. To stain for FAK or another marker that is detectable with antibodies, skip to Step 5.
4. Aspirate the nuclear labeling solution and wash the cell-containing gels three times with 1X PBS as in Step 2. Stained samples can be stored in 1X PBS at 4°C until further use.

5. **To stain for a protein of interest:** Aspirate the PBS from the tissue culture dishes, add 300 µL of 0.3% PBST, and incubate the sample at room temperature for 15 minutes.

6. Aspirate the PBS, cover the gels with blocking buffer, and incubate on a shaker (200 rpm) at room temperature for ~4 hours.

7. Aspirate the blocking buffer, cover the gels with primary antibody solution, and incubate on a shaker at 200 rpm overnight at 4°C.

8. Aspirate the primary antibody solution, add 0.3% PBST solution, and incubate on a shaker at 200 rpm at room temperature for 30 minutes. Aspirate the PBST and repeat every 30 minutes for 3-4 hours.

9. Repeat Steps 7 and 8, this time incubating with the secondary antibody solution. Wrap the tissue culture dishes with aluminum foil to prevent photobleaching of the secondary antibody. After the final wash, stained samples can be stored in 1X PBS at 4°C until further use.

10. To visualize samples, use a 10×/0.30 NA objective focused on the midplane of the epithelial tissues.

11. To visualize cell nuclei, image fixed samples labeled with a nuclear marker using a 10×/0.30 NA objective under UV illumination.

12. To visualize samples stained for a protein of interest, image using a 10×/0.30 NA objective on an inverted fluorescence microscope.
13. To create frequency maps of protein staining of multiple tissues (typically 50 or more) of identical initial geometry, first import each of the images using standard image analysis software (see Materials above) and convert them to 8-bit grayscale. To threshold these images, convert them to binary (i.e. halftone/black and white) by defining a grayscale cutoff point; grayscale values below the threshold become black, and those above the threshold become white. Then, combine each of the individual binary images into a single image stack.

14. Next, register the stacked images (i.e. align or match them) in the analysis software. Many image analysis software packages come with a freely available registration plugin. Essentially, each image in a stack is used as a template for the alignment of the next image, such that the images in the entire stack are aligned by propagation. Finally, overlay (project) the images in the aligned image stack based on average intensity to form a single image with a pixel frequency map. This image can be color-coded using image editing software of choice [167, 168].
Appendix B

3D TFM of Engineered Epithelial Tissues

This appendix is adapted from the following publication:


Several biological processes, including cellular migration, tissue morphogenesis, and cancer metastasis, are fundamentally physical in nature; each implicitly involves deformations driven by mechanical forces. TFM was initially developed to quantify the forces exerted by individual isolated cells in 2D culture. Here, we extend this technique to estimate the traction forces generated by engineered 3D epithelial tissues embedded within a surrounding ECM. This technique provides insight into the physical mechanisms that underlie tissue morphogenesis in 3D.
B.1 Introduction

B.1.1 History and importance of TFM

Many fundamental biological processes are driven by an interplay between mechanical and biochemical signals, including cell proliferation [281], differentiation [282, 283, 284], and EMT [143, 144]. Quantifying the mechanical forces that cells exert, however, and assessing their role in the vast array of molecular signaling networks that regulate cell behavior is a tremendous challenge. This is partially owing to the fact that mechanical loads act across hierarchical length scales (e.g., should we be measuring forces at the level of the cytoskeleton, the level of the organism, or somewhere in between?). In addition, cells are usually ill-suited to traditional mechanical tests, as they are small and often mechanically inaccessible, both when embedded in a dense meshwork of ECM and when interconnected with neighboring cells in an intact epithelium.

Still, despite these challenges, significant progress has been made in the study of mechanical forces in biology with the advent of TFM. This technique involves cells (or tissues) cultured on or embedded within a flexible substratum that can deform in response to cell-generated tractions. If the mechanical properties of the substratum are well characterized, then the traction forces that generated the observed deformations can be calculated (see Note 1).

When TFM was first invented, deformations were restricted to 2D, since cells were typically cultured on thin films [285, 286, 287, 288]. TFM was later expanded to 3D systems; first, by accounting for 3D deformations of the underlying substratum during 2D cell culture [289], and then later by studying individual cells embedded in 3D gels [290]. Single cells have been estimated to exert forces on the order of 10 nN on their surrounding substrata [289].
B.1.2 Recent contributions

We were among the first to fully extend 3D TFM into a multicellular context. Engineered mammary epithelial tissues can be embedded within 3D collagen gels \cite{230} containing fluorescently labeled polystyrene microspheres, which are used to track the deformations of the gel during culture and thereby estimate the traction stresses exerted by the developing tissues \cite{165}. Here we present a detailed protocol for these experiments, which can be readily extended to other types of cells, gels, and tissues.

B.2 Materials

B.2.1 PDMS stamps

1. Polydimethylsiloxane (PDMS) (Sylgard 184, Ellsworth Adhesives)
2. PDMS curing agent (Sylgard 184, Ellsworth Adhesives)
3. Lithographically patterned silicon master
4. Plastic weigh boat
5. 100-mm Petri dishes (Fisher Scientific)
6. 70\% (v:v) Ethanol
7. Razor blade

B.2.2 Micropatterning materials

Prepare collagen mixture on ice. Keep reagents at 4°C.

1. 10x Hank’s balanced salt solution (HBSS)
2. Cell culture media: 1:1 Dulbecco’s Modified Eagle’s Medium : Ham’s F12 Nutrient Mixture (DMEM/F12 (1:1), Hyclone) supplemented with: 2% fetal bovine serum (FBS), 5 µg/mL insulin, and 50 µg/mL gentamicin

3. Phosphate-buffered saline (PBS)

4. 0.1 N NaOH

5. Bovine type I collagen (non-pepsinized; Koken, Tokyo, Japan)

6. 1-µm diameter fluorescent polystyrene beads (Invitrogen)

7. 1% (m:v) bovine serum albumin (BSA) in PBS. Store at 4°C.

8. Curved stainless steel tweezers (#7 Dumont)

9. 35-mm tissue culture dishes (Fisher Scientific)

10. 15 mL conical tube (Fisher Scientific)

11. 1.5 mL Eppendorf Safe-Lock Tube (Eppendorf)

12. Circular #1 glass coverslips, 15-mm in diameter (Bellco Glass Inc)

13. Vybrant DiI (or DiO) cell-labeling solution (Invitrogen)

14. 0.05% 1X Trypsin-EDTA (Invitrogen)

15. 70% (v:v) Ethanol

16. Ice

**B.2.3 Confocal fluorescence microscopy**

1. CCD camera attached to an inverted spinning disk confocal microscope

2. 0.05% Triton X-100 in PBS
B.2.4 Tracking bead displacements

1. Imaris, Version 7.6.3 (Bitplane)

B.2.5 Calculating average displacement fields

1. MATLAB (The Mathworks)

B.2.6 Reconstructing tissue geometry

1. Inventor Professional (AutoDesk, Inc.)

B.2.7 Computing traction forces

1. COMSOL Multiphysics, Version 4.2a (COMSOL AB)

B.3 Methods

Here, we describe a 3D engineered tissue model used to quantify the traction forces exerted by tissues on their surrounding ECM (Figure B.1). Collagen matrices containing fluorescent beads with multiple tube-shaped cavities of defined geometry are created using a microlithography-based technique. The cavities are then seeded with epithelial cells labeled with fluorescent vital dye (DiI). The tissues and surrounding fluorescent beads are then imaged in 3D using confocal fluorescence microscopy both before and after treatment with Triton X-100. Bead displacements are tracked in 3D, and the resultant traction stresses are calculated using computational modeling.

B.3.1 Preparation of PDMS stamps for 3D micropatterning

1. Mix the PDMS prepolymer and curing agent at a 10:1 (w:w) ratio. Aim for a total weight of approximately 60 g. Remove the entrapped air bubbles by degassing in a
Cast PDMS stamp off silicon master
Coat stamp with BSA solution
Wash stamp with cell media
Mold collagen gel with embedded fluorescent beads
Seed gel with suspension of cells
Wash off excess cells
Place collagen lid on sample
Image confocal stack, relax tissues, and image post-relaxation confocal stack
Remove stamp from gel
Track bead displacements and estimate traction forces using COMSOL

Figure B.1: Schematic outlining the microfabrication procedure.

vacuum chamber (∼15 minutes). Pour the bubble-free mixture onto a lithographically patterned silicon master in a large weigh boat. Cure the PDMS in an oven at 60°C for at least 2 hours.

2. Once the PDMS is cured, remove the rims from the weigh boat and carefully peel the PDMS from the silicon wafer, removing any PDMS on the bottom of the master. Using a clean razor blade, cut off the excess PDMS from around the imprinted features, leaving the remaining PDMS in a circular shape.
3. Using a clean razor blade, cut the polymerized PDMS into stamps (∼5-mm cubes), making one stamp for each sample. Place the stamps feature-side-up in a 100-mm Petri dish.

4. Create supports for the PDMS stamps by spreading 2-3 g of PDMS on a 100-mm Petri dish using a spin coater (see Note 2). Cure the PDMS as described above. Using a razor blade, cut out two (∼5-mm square) supports per PDMS stamp and place the supports in the Petri dish containing the PDMS stamps.

5. In a biosafety cabinet (cell culture hood), sterilize stamps and supports by washing briefly with 70% ethanol and aspirating the excess liquid. Allow residual ethanol to evaporate completely (∼2 minutes).

### B.3.2 Micropatterning of 3D epithelial tissues

1. In a biosafety cabinet, coat four PDMS stamps with approximately 200 µL of 1% BSA in PBS (see Note 3). Leave the BSA-coated stamps at 4°C for a minimum of 4 hours to eliminate air bubbles.

2. Aspirate BSA from the four PDMS stamps.

3. Add cell culture media to the stamp surfaces, aspirate, and repeat (200 µL per four stamps per wash should be sufficient).

4. Using curved stainless steel tweezers, place the PDMS supports in the 35-mm tissue culture dishes (two supports per stamp, separated by a distance slightly less than the length of the stamp).

5. Wash four circular glass coverslips (15 mm in diameter, #1) with 70% ethanol and aspirate the excess liquid. Place these in a 100-mm Petri dish.

6. In a chilled 1.5 mL eppendorf tube, prepare a neutralized solution of collagen. Add 50 µL 10x HBSS buffer, 30 µL 0.1 N NaOH, 30 µL cell culture media, and 400 µL of
stock collagen for a final concentration of 4 mg/mL (see Note 4). Mix slowly by pipeting up and down; try not to introduce bubbles. If bubbles are introduced, centrifuge the mixture. If fluorescent beads are to be used for TFM, add them to the collagen mixture at this time at a high concentration (∼4 × 10^8 beads/mL).

7. Distribute 200 µL of the collagen mixture evenly on the surfaces of the PDMS stamps (approximately 50 µL per stamp).

8. Using the curved tweezers, flip the stamps upside down onto the supports in the tissue culture dishes and place the dishes into the incubator at 37°C for approximately 30 minutes. Add 50 µL of the collagen mixture onto each of the circular coverslips (these will be the collagen caps that are placed on top of the patterned tissues) and place these into the incubator at 37°C for approximately 30 minutes.

9. Aspirate the media from a 100-mm tissue culture dish that is 40% confluent with epithelial cells. Add 10 µL Vybrant DiI (or DiO) (Invitrogen) in 2 mL of fresh culture media to the tissue culture dish and incubate at 37°C for 15 minutes.

10. After the incubation, aspirate the media containing DiI or DiO and trypsinize the cells using 2 mL of trypsin.

11. Add 8 mL of cell culture media to the trypsinized cells, place in a 15 mL conical tube and centrifuge at 800 rpm for 5 minutes.

12. After the centrifugation step, aspirate the supernatant and resuspend the cells in 250–400 µL of cell culture media for a final cell concentration of ∼10^6–10^7 cells/mL (see Note 5).

13. Remove the tissue culture dishes containing the PDMS stamps and collagen from the incubator and lift the PDMS stamps off of the collagen using the curved tweezers (see Note 6); the stamps can now be discarded.
14. Add 30 µL of cell suspension onto each collagen gel. While observing under a microscope, shake the dishes so that the cells settle into the patterned collagen (see Note 7).

15. After ∼5 minutes (or whenever the cells have settled into the wells of the pattern), wash the stamps with 430 µL of cell culture media by tilting the tissue culture dish on its side and allowing the media to pour over the stamp (see Note 8). Aspirate the wash from the tissue culture dish and repeat.

16. Place the collagen containing the cells in the incubator at 37°C for 15 minutes. Then, place the glass coverslips with collagen caps on top of the cell-containing gels so that the cells are completely embedded in collagen (Figure B.2). Place the dishes in the incubator at 37°C again for 15 minutes.

17. Add cell culture media slowly on top of the glass coverslip until the cell-containing gels are covered in media, and place the dishes in the incubator at 37°C.

![Figure B.2: Micropatterned epithelial tissues. (A) Bright field image of micropatterned epithelial tissues. (B) Higher magnification view of micropatterned tissue presented in (A). (C) Confocal fluorescence image (maximum z-intensity) of tissue shown in (B); 1 µm diameter fluorescent microspheres (green), DiI-labeled cells (red). Scale bars = 50 µm.](image-url)
B.3.3 Confocal timelapse imaging of fluorescent bead displacements and tissue morphogenesis

1. Monitor the positions of the fluorescent beads by collecting confocal stacks of the tissues: 120 images (spaced 1 µm apart).

2. Relax the tissues by adding 0.05% Triton X-100 in PBS overnight.

3. Capture a second stack of post-relaxation images (120 images, 1 µm apart) of fluorescent beads the next day (see Note 9).

4. To account for experimental noise in the motion of the fluorescent beads, monitor the positions of the beads in cell-free collagen gels (see Note 10).

B.3.4 Tracking bead displacements

1. For each tubule, import 3D image stacks into Imaris (see Note 11). There should be two time points before and after treatment with Triton X-100.

2. Correct for rigid body drift between image stacks.
   
   (a) Select Edit → Properties, and input voxel dimensions.
   
   (b) In Surpass view, use Spots filtering to select a region of beads far away from the tissue.

   (c) Track these spots using the Autoregressive Motion routine.

   (d) Highlight all tracks in the ‘Edit Track’ window and select ‘Correct Drift.’ This should be largely rigid body motion (i.e. only translations and rotations).

   (e) In Surpass view, the newly drift-corrected image stack should appear.

3. Quantify 3D bead displacements in the entire imaging volume using the Spots filter and the Autoregressive Motion tracking routine (Figure B.3) (see Note 12).
4. Export the tracked displacements as a spreadsheet. The following quantities will be used for further analysis:

<table>
<thead>
<tr>
<th>Track Position Start X</th>
<th>Track Position Start Y</th>
<th>Track Position Start Z</th>
<th>Track Displacement X</th>
<th>Track Displacement Y</th>
<th>Track Displacement Z</th>
</tr>
</thead>
</table>

Figure B.3: Tracking bead displacements. (A) Maximum z-intensity image of confocal stack showing fluorescent microspheres before treatment with Triton X-100; the epithelial tissue outline is indicated with a white dashed line. (B) Maximum z-intensity image of confocal stack showing fluorescent microspheres after treatment with Triton X-100; bead displacements are shown in purple, and the tissue outline is indicated with a white dashed line. The inset shows a higher magnification view of the boxed region. (C) 3D reconstruction of bead trajectories shown in (B) with indicated dimensions. Scale bars = 50 µm.
B.3.5 Computing average displacement field (using data from multiple tissues)

1. Use the *griddata* subroutine in MATLAB to interpolate the exported displacement data across a 3D grid spanning the entire imaging volume. Use Track Position Start X, Y, Z to define a point cloud, then interpolate each component of the displacement field (e.g., Track Displacement X) separately. These data can often be fairly noisy (Figure B.4A).

2. To increase the signal to noise ratio, each component of the displacement field can be averaged across multiple tissues (Figure B.4B, C), but only if the displacement data from multiple tissues are properly aligned using the fluorescently labeled tissue geometry. Especially for small displacements, subtle alignment errors can produce significant artifacts in the averaged displacement field.

3. Ensure the data for each component of the displacement field is organized in a format compatible with the finite element package used to compute the resultant traction forces.

B.3.6 Reconstructing and exporting tissue geometry

1. Using Imaris, measure average morphological parameters (e.g., tissue length, height, etc.) for several fluorescently labeled tissues (Figure B.5A).

2. Use the measured parameters to draw a 3D surface in AutoDesk Inventor Professional. Save the reconstructed geometry as an Inventor .ipt file (Figure B.5A).
Figure B.4: Displacement fields around engineered tissues. (A) Plot of the interpolated total displacement field for an individual tissue. (B) Plot of the interpolated total displacement field averaged over 20 tissues. (C) Plot of the averaged x-component of the displacement field.

### B.3.7 Calculating traction forces

1. Construct a 3D finite element model to compute the traction forces exerted by the engineered tissues. Although other commercial software packages can be used, the following protocol employs COMSOL Multiphysics Version 4.2a (see Note 13).

   (a) Open COMSOL Multiphysics and create a New (File → New...) model file.

   (b) Select ‘3D’ for Space Dimension, ‘Solid Mechanics (solid)’ for Add Physics, and ‘Stationary’ for ‘Select Study Type’ (see Note 14). Click the ‘Finish’ icon.
Figure B.5: Computing traction forces. (A) Tissue geometry was reconstructed in AutoDesk Inventor from measurements of fluorescently-label tissues in Imaris. Adapted from [165]. (B) Model geometry used to compute traction forces in COMSOL Multiphysics. (C) Finite element mesh. (D,E) Imported displacement field prescribed as boundary condition along surface of imported tissue geometry. Here, the x-component of the displacement field ‘displx’ is shown. (Compare to Figure B.4C.) (F) Model-computed traction stresses.

2. Create model geometry

(a) Click ‘Geometry 1’ and specify the appropriate units for length.

(b) Right-click ‘Geometry 1’ in the Model Builder window, and click ‘Import’. Under the ‘Geometry import:’ pull-down menu, select ‘3D CAD file’, then click ‘Browse...’ to locate the Inventor .ipt file containing the exported tissue geometry, and click ‘Import’ (see Note 15). Ensure that ‘Solids’ and ‘Surfaces’ are checked under ‘Objects to import’ and that ‘Form solids’ is selected under the ‘Import options’ pull-down menu. (If the length units specified in the CAD file are correct, ensure that ‘From the CAD document’ is selected from the ‘Length unit’ pull-down menu.)
(c) Right-click ‘Geometry 1’ in the Model Builder window, and click ‘Block’. Under the ‘Size and Shape’ menu, select values for ‘Width,’ ‘Depth,’ and ‘Height’ that are significantly larger than that of the imported tissue geometry. This block will represent the surrounding collagen gel. (The appropriate size will depend on the how far the displacement field propagates into the gel. The displacements should all decay to zero before the outer boundary of the block. Here, we specified a cube with sides of 1 mm.) Click the ‘Build Selected’ icon.

(d) Right-click ‘Geometry 1’ in the Model Builder window, and under the ‘Transforms’ menu, click ‘Move’. Under ‘Input objects:’, click on the imported tissue geometry and click the ‘+’ icon. Select ‘imp1’ (i.e., the imported tissue geometry) and specify values for ‘x,’ ‘y,’ and ‘z’ that move the tissue to a location within the surrounding gel that will coincide with the position of the tissue in the measured displacement field exported from Imaris. (The two must be aligned to ensure that the correct experimental displacements are interpolated along the model tissue surface.)

(e) Right-click ‘Geometry 1’ in the Model Builder window, and under the ‘Boolean Operations’ menu, click ‘Difference’. Under ‘Objects to add’, click on the block and click the ‘+’ icon. Under ‘Objects to subtract’, click on the imported tissue geometry and click the ‘+’ icon.

(f) Right-click ‘Geometry 1’ in the Model Builder window, and click ‘Build All’. This final geometry (Figure B.5B) represents the geometry of collagen gel surrounding the tissue.

3. Import the (averaged) experimental displacements

(a) Right-click ‘Global Definitions’ in the Model Builder window, and under the ‘Function’ menu, select ‘Interpolation’.
(b) Under ‘Data Source’, select ‘File’ and click ‘Browse...’ to locate the data file containing the x-component of the averaged gel displacements. (Ensure that the units of the grid points specified in the imported displacements file match the units in the model.)

(c) Select the appropriate data format and assign the function a name. (Here we use ‘displx’). Check the box for ‘Use space coordinates as arguments’. Under the Extrapolation pull-down menu, select ‘Specific value’ and input ‘0’ for ‘Value outside range’.

(d) Repeat and create separate interpolation functions for the y- and z-components of the averaged gel displacements. (Here we name these functions ‘disply’ and ‘displz’, respectively.)

(e) Right-click ‘Definitions’ under ‘Model 1’ in the Model Builder window, and select ‘Variables’. Select ‘Boundary’ from the ‘Geometric entity level’ pull-down menu. Ensure ‘Manual’ has been selected from the ‘Selection’ menu.

(f) Select the surfaces bounding the imported tissue geometry and define the following variable:

\[
\text{tract} = \sqrt{\text{solid.Tax}^2 + \text{solid.Tay}^2 + \text{solid.Taz}^2}
\]

4. Define mechanical properties and boundary conditions

(a) Expand ‘Solid Mechanics (solid)’ in the Model Builder window, and click ‘Linear Elastic Material Model 1’.

(b) Ensure the entire model geometry is selected. Under the ‘Linear Elastic Model’ menu, select ‘Isotropic’ and specify the Young’s modulus and Poisson’s ratio as follows (see Note 16):

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)</td>
<td>Young’s modulus</td>
<td>User defined</td>
<td>750 Pa</td>
</tr>
<tr>
<td>(\nu)</td>
<td>Poisson’s ratio</td>
<td>User defined</td>
<td>0.2</td>
</tr>
</tbody>
</table>
(c) Right-click ‘Solid Mechanics (solid)’ and select ‘Prescribed Displacement.’ Under ‘Boundary Selection’, ensure ‘Manual’ is selected, and select the surfaces bounding the tissue by clicking on them individually and selecting ‘+’ (Figure B.5D, E).

i. Check the box next to ‘Prescribed in x direction’ and input the following (see Note 17):

\[
\begin{array}{c|c}
  u_0 & -\text{displx}\times10^{-6} \\
\end{array}
\]

ii. Repeat for displacements in the y- and z-directions, using ‘disply’ and ‘displz’, respectively.

(d) Right-click ‘Solid Mechanics (solid)’ and select ‘Fixed Constraint.’ Under ‘Boundary Selection’, ensure ‘Manual’ is selected, and select each of the outer surfaces of the box representing the collagen gel.

5. Create model mesh

(a) Right-click ‘Mesh 1’ in the Model Builder window. Select ‘Free Triangular’ under ‘More Operations’.

(b) Under ‘Geometric entity level’, ensure that ‘Boundary’ is selected from the pull-down menu and that ‘Manual’ is selected from the ‘Selection’ menu.

(c) Select the surfaces bounding the imported tissue geometry.

(d) Right-click ‘Free Triangular 1’ and select ‘Size’. Under the ‘Predefined’ list for ‘Element Size’, select ‘Fine’.

(e) Right-click ‘Mesh 1’, and select ‘Free Tetrahedral’. Ensure that ‘Remaining’ is selected as the ‘Geometric entity level’.

(f) Right-click ‘Free Tetrahedral 1’ and select ‘Size’. Under the ‘Predefined’ list for ‘Element Size’, select ‘Coarser’.

(g) Right-click ‘Mesh 1’ and select ‘Build All’ (Figure B.5C).
6. Solve model and plot results

(a) Right-click ‘Study 1’ in the Model Builder window and select ‘Compute’. Once the solution converges, results can be plotted in the ‘Results’ tab. (Ensure that the scale factor for deformations in all plots is set to 1.)

(b) To plot traction stresses, right-click ‘Results’ and create a ‘3D Plot Group.’ Right-click the ‘3D Plot Group’ and select ‘Surface’. Under ‘Expression’, type ‘tract’ (defined above) and click Plot (Figure B.5F).

(c) Ensure that model displacements reasonably reproduce those observed experimentally. If they do not, a more accurate description of the mechanical properties of the surrounding gel may be needed. Alternatively, nonlinear effects due to large deformations may likewise need to be included in the analysis.

B.4 Notes

1. It is important to note that forces are not measured directly here. Instead, they are calculated from the observed deformations of flexible substrata. The accuracy of the computed forces is thus highly dependent on the measured mechanical properties of these flexible materials, which are (typically) only characterized for a specific set of loading conditions. Moreover, simplifying assumptions such as material linearity, isotropy, and homogeneity are often assumed. These assumptions are often made for convenience and only approximate the actual behavior of real materials. The traction forces computed using TFM are thus only estimates.

2. The thickness of the supports will determine how close the epithelial tissues are to the bottom of the tissue culture dish.

3. In a biosafety cabinet, place 4 dots of BSA in the 4 corners of each stamp and one in the center, then spread with a pipet tip so that BSA covers the entire stamp.
4. When using a new bottle of collagen (5 mg/mL), check the pH of the final mixture. The pH should be 8-8.5; adjust the volume of NaOH accordingly.

5. Err on the side of less media; a more concentrated cell suspension will promote faster and more homogeneous settling of the cells into the collagen cavities.

6. Gently lift the PDMS stamps off the collagen as vertically as possible so as not to disturb the pattern.

7. To improve settling of the cells into patterned wells, place the tissue culture dishes next to a bench top centrifuge set to 700 rpm. The vibrations from the centrifuge shake the cells into the collagen cavities.

8. It may be easier to wet the bottom support first so that the wash flows straight down.

9. The elastic recoil happens quickly after adding the detergent (as soon as the detergent diffuses in and lyses the cells). Full relaxation/viscous flow is complete after treating with detergent overnight.

10. This accounts for noise owing to bead movement (which is negligible in these dense gels) as well as for inaccuracies in the Imaris tracking algorithm. In a single sample, signal-to-noise was typically 5:1, but averaging data from multiple samples attenuated the ratio to ∼20:1.

11. Other (free) tracking applications are available. Their implementation, however, requires that the user is proficient with different programming languages (e.g. MATLAB). Examples include Crocker and Grier’s 3D particle tracking algorithm [291], and Franck and colleagues digital volume correlation (DVC) [292].

12. Filters (using quantities such as Track Length) can be used to screen errant tracks.
13. Although the steps outlined in this protocol are specific to COMSOL Multiphysics Version 4.2a, a similar workflow can be developed in both older and newer versions of the software.

14. In selecting a ‘Stationary’ study type, we thereby neglect inertial effects, which are typically negligible in problems such as cell migration and tissue morphogenesis.

15. Several other CAD formats are compatible with COMSOL. If one would prefer to not use Inventor, consult the COMSOL Mulitphysics User’s Guide for a list of these alternatives [293].

16. As only a first approximation, we assume linear elastic material properties for the surrounding collagen gel [165].

17. In our analysis, displacements were interpolated in micrometers and therefore converted into meters. The negative sign is owing to the fact that displacements were tracked from before to after treatment with Triton X-100—that is, from the deformed to the undeformed configuration of the gel, which here must be reversed.
Appendix C

A 3D Culture Model to Study how IFP and Flow Affect the Behavior of Epithelial Cell Aggregates

This appendix is adapted from the following publication:


C.1 Materials

Prepare collagen mixture on ice. Keep reagents at 4°C.

C.1.1 Preparation of PDMS chamber and cavity surrounded by collagen

1. Polydimethylsiloxane (PDMS) (Sylgard 184, Ellsworth Adhesives)
2. PDMS curing agent (Sylgard 184, Ellsworth Adhesives)

3. Lithographically patterned silicon master

4. ¼”-hole punch

5. 150-mm petri dishes (Fisher Scientific)

6. 100-mm petri dishes (Fisher Scientific)

7. 100-mm tissue-grade polystyrene culture dishes (Fisher Scientific)

8. 24 mm x 50 mm #1½ glass coverslips (Fisher Scientific)

9. 18 mm x 18 mm #2 glass coverslips (Fisher Scientific)

10. 70% (v:v) ethanol

11. 0.12 mm x 30 mm acupuncture needles (Seirin)

12. 1.5 mL microcentrifuge tubes (Eppendorf)

13. 10x Hank’s balanced salt solution (HBSS)

14. 0.1 N NaOH

15. Bovine dermal type I collagen (non-pepsinized; Koken, Tokyo, Japan)

16. Cell culture medium: for example, 1:1 Dulbecco’s Modified Eagle’s Medium : Ham’s F12 Nutrient Mixture (DMEM/F12 (1:1), Hyclone) supplemented with: 10% fetal bovine serum (FBS), and 50 µg/mL gentamicin.

17. Sterile phosphate-buffered saline (PBS)

18. 1% (w:v) bovine serum albumin (BSA) in PBS. Store at 4°C.

19. Handheld drill (Dremel)
C.1.2 Formation of 3D epithelial cell aggregates under a controlled pressure profile

1. Culture medium (see Subheading 2.1, item 16)

2. 0.05% 1X Trypsin-EDTA (Invitrogen)

3. Collagen gels with channels, assembled between PDMS chambers and glass coverslips

4. PDMS (Sylgard 184, Ellsworth Adhesives)

5. PDMS curing agent (Sylgard 184, Ellsworth Adhesives)

6. 1/4”-hole punch

C.1.3 Immunofluorescence analysis

1. 16% paraformaldehyde (Electron Microscopy Sciences)

2. PBS

3. 1.5 mL microcentrifuge tubes (Eppendorf)

4. PBS with 0.3% (v:v) Triton X-100 (0.3% PBST)

5. Normal goat serum

6. Rabbit anti-E-cadherin antibody (Cell Signaling)

7. Alexa 488 goat anti-rabbit antibody (Invitrogen)

8. Nuclear counterstain, such as Hoechst 33342 (Invitrogen)

9. Aluminum foil
C.1.4 Imaging equipment

1. Inverted microscope with phase-contrast and fluorescence capabilities and a 10X/0.30 NA objective

C.1.5 Image analysis: measuring the extent of collective migration

1. ImageJ (National Institutes of Health, Bethesda, MD)

C.2 Methods

Here we describe an engineered 3D culture model that can be used to study the effects of pressure gradients and fluid flow on the migratory/invasive behavior and gene expression profile of an aggregate of epithelial cells.

C.2.1 Preparation of PDMS chamber and cavity surrounded by collagen

1. Mix the PDMS prepolymer and curing agent at a 10:1 (w:w) ratio. Aim for a total weight of approximately 50 g. Remove the entrapped air bubbles by degassing in a vacuum chamber (∼15 minutes). Pour the bubble-free mixture onto a lithographically patterned silicon master in a 150-mm petri dish. The silicon master should have features that produce channels that are approximately 20 mm long, 1 mm wide, and 1 mm tall spaced approximately 1.5 cm apart. Cure the PDMS in an oven at 60°C for at least 2 hours.

2. Once the PDMS is cured, carefully peel the PDMS from the silicon wafer, removing any PDMS from the bottom of the master. Using a clean razor blade, cut off the excess PDMS from around the molded features.
3. Using a clean razor blade, cut the polymerized PDMS into chambers containing individual channels ~1.25 cm wide and ~2.5 cm long (Figure 2.5A). Use a 1/4"-hole punch to bore holes on either side of each channel well. Bore one of the holes (hole B in Figure 2.5B) in the middle of the channel such that the distance between the holes is 8-10 mm. Sterilize the chambers carefully in a biosafety cabinet (cell culture hood) by sonicating the chambers in 70% ethanol, washing briefly with 100% ethanol, and aspirating the excess liquid (see Note 1).

4. Drill a rectangular hole (~15 mm × ~40 mm) in the middle of a 100 mm tissue culture dish.

5. In a biosafety cabinet, wash the modified tissue culture dish containing the rectangular hole with 100% ethanol to sterilize.

6. In a biosafety cabinet, carefully sterilize one 24 mm × 50 mm #1 1/2 glass coverslip and two 18 mm × 18 mm #2 glass coverslips by sonicating in 70% ethanol, washing briefly with 100% ethanol, and aspirating the excess liquid.

7. Lay down the 18 mm × 18 mm #2 glass coverslips parallel to the ~15 mm sides of the hole in the 100 mm tissue culture dish, leaving a few mm of space between the coverslips and the hole (Figure 2.5C).

8. Mix the PDMS prepolymer and curing agent and remove air bubbles as described previously. Aim for a total weight of approximately 5 g. Using a 200 µL pipet tip, add a layer of PDMS on top of the modified 100 mm tissue culture dish around the hole and between the hole and the coverslips. Then, carefully lay the 24 mm × 50 mm #1 1/2 coverslip on top of the two 18 mm × 18 mm #2 coverslips such that it covers the hole forming a seal with the PDMS. Cure the PDMS in an oven at 60°C for at least 2 hours.
9. Mix the PDMS prepolymer and curing agent as described previously. Aim for a total weight of approximately 20 g. Again, remove the entrapped air bubbles by degassing in a vacuum chamber (∼15 minutes). Using a pipette tip, paint a stripe of PDMS in a straight line ∼1.5 cm from the edge of a 100 mm petri dish. Lay down 12-14 120 m diameter acupuncture needles with handles placed on the PDMS (Figure 2.5D). Cure the PDMS in an oven at 60°C for at least 1 hour.

10. Pour the remainder of the 20 g PDMS mixture on top of the needles. Cure the PDMS in an oven at 60°C for at least 2 h.

11. Using a clean razor blade, separate the individual needles embedded within PDMS and remove a portion (∼1 cm) of the handles. Use the razor blade to cut away the PDMS surrounding the needle, leaving a small rectangular portion in the middle of the needle to be used for support (Figure 2.5E). Sterilize the needles carefully in a biosafety cabinet by sonicating in 70% ethanol, washing briefly with 100% ethanol, and aspirating the excess liquid.

12. Coat the needles with 1% BSA in PBS for at least 4 hours at 4°C and then wash with PBS and ddH₂O.

13. In a biosafety cabinet, place three of the PDMS chambers in the middle of one modified glass-bottom 100-mm tissue culture dish such that the channels are parallel to one another (and perpendicular to the 50 mm sides of the 24 mm × 50 mm #1½ glass coverslip) with the open face of the channels against the glass (see Note 2). Carefully thread the cleaned needles into the side of the chamber next to hole B such that the tip of the needle is in between the two wells formed by holes A and B and in the middle of the channel (Figure 2.5F). Secure the needle in place by conformally adhering the PDMS support to the tissue culture dish next to the PDMS chamber and the 24 mm × 50 mm #1½ glass coverslip. Cool the tissue culture dishes to 4°C for at least 2 hours (see Note 3).
14. In a cold (4°C) 1.5 mL microcentrifuge tube, prepare a neutralized solution of collagen. Add 30.6 µL 10x HBSS, 18.4 µL 0.1 N NaOH, 244.8 µL collagen, and 12.2 µL cell culture medium for a final concentration of collagen of approximately 4 mg/mL (see Note 4). Mix slowly by pipeting up and down; try not to introduce bubbles. If bubbles are induced, centrifuge the mixture briefly at 13,000 rpm.

15. Add 15 µL of neutralized collagen solution to the well formed by hole A (well A) of the PDMS chamber, opposite to the side containing the needle (Figure 2.5F). Tilt the culture dish on its side to allow the collagen to flow down and fill the channel, tapping the dish as necessary (see Note 5). Gently aspirate the excess collagen from well A. Incubate at 37°C for 20 minutes.

16. Add 20 µL of cell culture medium to both wells at the gel surface in order to wet the gel. Gently remove the needle from the chamber: bend it 90° where it exits the PDMS support, and gently pull it straight out while holding down the PDMS support.

17. Add a small amount of PDMS (prepared as above and incubated at 60°C for 15 minutes) to plug the channel next to the well formed by hole B (well B) that contained the needle (Figure 2.5G). Add 20-50 µL of cell culture medium to both wells once the PDMS is cured. Incubate the channels overnight at 37°C.

C.2.2 Formation of 3D epithelial cell aggregates

1. Aspirate the cell culture medium from the wells in the PDMS chamber.

2. Trypsinize epithelial cells (in this case MDA-MB-231 human mammary carcinoma cells) and resuspend in cell culture medium at a final concentration of approximately 10^7 cells per mL.

3. Add 50 µL of the concentrated suspension of cells to well B and allow the cells to fill the cavity by convection (Figure 2.6A, B).
4. Resuspend the cells in well B after 2-5 minutes. Then, once the cavity is completely filled, aspirate the cell suspension from the well.

5. Wash well B twice with 50 µL of medium.

6. Add fresh medium to the rim of well A and slightly over the rim of well B. Incubate the seeded channels at 37°C for 48 h, changing the medium every 24 hours.

7. After 48 hours, discard any samples in which cells have migrated outside of the original shape of the aggregate.

8. Mix the PDMS prepolymer and curing agent as described above. Aim for a total weight of approximately 50 g. Pour the bubble-free mixture onto a 150 mm petri dish. Cure the PDMS in an oven at 60°C for at least 1 hour.

9. Using a clean razor blade, cut the PDMS into blocks that are approximately 1 cm by 1 cm. Use a 1/4”-hole punch to bore a hole in the center of the blocks, creating PDMS gaskets (Figure 2.6C).

10. To set up a hydrostatic pressure differential across the cell aggregates in the collagen channels within the PDMS chambers, add up to four PDMS gaskets on top of one of the wells on one side of the channel. Ensure that the holes in the PDMS gaskets align with the wells in the chamber (Figure 2.6D). Seal the PDMS gaskets conformally to the chamber by gently pressing down.

11. Add culture medium to the rim of both wells. Maintain the pressure differential by replenishing the medium on the higher pressure side every 12 hours (see Note 6).

12. Culture the cell aggregates for up to 9 days at 37°C.

**C.2.3 Immunofluorescence analysis**

1. Prepare a fixative by diluting 16% paraformaldehyde 1:4 (v:v) in PBS.
2. Aspirate the medium from the PDMS chamber and add fixative to well B until the well is slightly overfilled. Incubate at room temperature for ~18 minutes.

3. Aspirate the fixative and fill well B to the rim with PBS. Repeat twice, each after 20 minutes, for three total washes.

4. **To stain for nuclei:** prepare 4 mL of a 1:1000 (v:v) solution of Hoechst 33342 in PBS. To stain for E-cadherin or another marker that is detected with antibodies, skip to Step 8.

5. Aspirate the PBS from well B and fill it to the rim with the Hoechst solution. Incubate at room temperature for 15-20 minutes.

6. Aspirate the Hoechst solution and wash well B three times with PBS as in Step 3. Stained samples can be stored in PBS at 4°C until further use.

7. If desired, visualize the stained nuclei as described in Section C.2.4.

8. **To stain for E-cadherin or other protein marker (Figure 2.7):** aspirate PBS. Peel the PDMS chamber from the tissue culture dish. Carefully remove the collagen channel and embedded cell aggregate from the underside of the PDMS chamber and place in a 1.5 mL microcentrifuge tube (see Note 7).

9. Add 300 µL of PBST to the tube and incubate the sample at room temperature for 15 minutes.

10. Prepare blocking buffer by diluting goat serum 1:10 (v:v) in PBST.

11. Remove the sample and place in a new 1.5 mL microcentrifuge tube. Add 300 µL of blocking buffer and incubate on a shaker at room temperature for several hours.

12. Prepare a 1:200 (v:v) solution of primary rabbit anti-mouse E-cadherin antibody in blocking buffer (see Note 8).
13. Remove the sample and place in a new 1.5 mL microcentrifuge tube. Add 300 \( \mu \text{L} \) of the primary antibody solution and incubate on a shaker overnight at 4°C.

14. Remove the sample and place in a new 1.5 mL microcentrifuge tube. Add 300 \( \mu \text{L} \) of PBST and incubate on a shaker at room temperature for 30 minutes. Repeat with a fresh microcentrifuge tube and PBST aliquot every 30 minutes for 3-4 hours.

15. Repeat steps 12-14 with the secondary Alexa 488 goat anti-rabbit antibody. Wrap the microcentrifuge tubes with aluminum foil to prevent photobleaching of the secondary antibody. After the final wash, stained samples can be stored in PBS at 4°C until further use (see Note 9).

16. Visualize samples as described in Section C.2.4.

C.2.4 Imaging techniques

1. To image samples, use a 10X/0.30 NA objective focused on the midplane of the tip of the epithelial cell aggregate (see Note 10).

2. To monitor cell migration over time in the culture model under various flow conditions, capture phase-contrast images of the epithelial cell aggregates (Figure 2.8A; Figure 2.9A) on each day for up to nine days using an inverted phase-contrast microscope.

3. To image stained samples, transfer the samples onto a glass slide, and place a drop of PBS on top of each sample to keep the sample hydrated.

4. To visualize cell nuclei, image fixed Hoescht 33342-stained samples under UV illumination (Figure 2.8B; Figure 2.9B).

5. To visualize samples stained for E-cadherin, image using an inverted fluorescence microscope (Figure 2.8C).
C.2.5 Image analysis: measuring the extent of invasion

1. Open the phase-contrast image files in ImageJ.

2. Set the scale of the image according to microscope calibrations by selecting ‘Set Scale...’ under the ‘Analyze’ menu.

3. Using the line tool on the main menu, draw a line along the length of a collectively migrating cohort protruding from the aggregate (Figure 2.9C) (see Note 11).

4. Measure the length of the line by clicking ‘Measure’ under the ‘Analyze’ menu. This will output the length of the line in the units specified (Figure 2.9D).

C.3 Notes

1. Cleaning should eliminate all leftover debris and dust particles.

2. Press down on the PDMS chambers to ensure that they are conformally adherent to the tissue culture dish.

3. The PDMS chambers must be chilled prior to the addition of collagen to prevent premature gelation.

4. When using a new bottle of collagen, check the pH of the final mixture. We used a pH of 8.5-9. To alter the pH, adjust the volume of NaOH accordingly.

5. To help the collagen mixture flow into the channels, turn the tissue culture dish on its side (with the channels perpendicular to the work surface) while tapping.

6. The hydrostatic pressure differential across the aggregate can range from 0.4 to 1.6 cm H$_2$O depending on how many PDMS blocks are used. Interstitial flow velocities are on the order of 1 µm/s (average flow rates of 20-100 µL/day).
7. Remove the collagen channel containing the epithelial cell aggregate from the PDMS chamber with forceps. Be sure to grip the channel from the end adjacent to well A.

8. Here, we stained for E-cadherin, but the same protocol may be used for other primary antibodies.

9. In addition to staining, one can also use real time RT-PCR analysis to quantify changes in gene expression. For this analysis, it is necessary to combine at least six samples per condition to obtain an adequate amount of RNA. Samples must also be incubated with collagenase (we recommend a 2 mg/mL solution of collagenase from Clostridium histolyticum (Sigma) in culture medium) prior to RNA extraction using an RNeasy Mini Kit (Qiagen).

10. To visualize 3D features of the mammary epithelial cell aggregates (live or fixed), capture confocal stacks of the samples using an inverted spinning disk confocal microscope (200 images, 1 μm apart).

11. We defined collective migration as protrusions from the primary aggregate, still attached to the latter, containing multiple nuclei.
Bibliography


