Investigating the role of spatial presentation of physical and chemical cues in regulating normal and cancer cell polarity

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Dedication

This thesis is dedicated to my nephews, Caleb and Ryan, for their unending curiosity, joy of discovery and gleeful enthusiasm for anything that can be called an “Experiment.”
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Abstract of Dissertation

Throughout the body, different cell types are required to move in order to perform their normal physiological functions. Immune cells constantly patrol the body in search of infections. Epidermal cells remain stationary until required to move to heal a nearby wound. Cells in hair follicles or intestinal crypts continuously migrate outward to replace routine losses. These processes all require cells to move at a specific rate and in a given direction appropriate to their task and environment. Sometimes, the cellular programming that dictates these movements malfunctions, leading to disease. Preventing these diseases or enhancing these functions requires a fundamental understanding of cell migration.

Placing cells in flat, plastic dishes and analyzing their movement, has led to a great many discoveries about the mechanics of how cells move. However, the same lack of environmental features that enables studies of unbiased cell movement, makes it challenging to study how cells migrate in response to external mobilizing signals. On the other extreme, watching cells move in an intact organism, such as axon growth in *C. elegans* or border cell migration in *Drosophila*, runs the risk of having too many external signals to account for.

To address this gap, we have developed an experimental platform that enables select soluble cues to be presented to cells in a controllable fashion, such as a gradient. The substrate of the platform is modified to present patterns of physical cues at well-defined orientations to the soluble cue. This system allows the application of multiple, physiologically relevant cues to cells while minimizing external influences and
maintaining the accessibility and ease of measurement common to traditional cell culture dishes.

This platform was applied to the study of cell polarity and motility in normal and cancerous cells by combining gradients of chemotactic factors and patterns of adhesive extracellular ligands. We find that even in this minimalist system, unexpected factors bias the polarity of stationary cells on micropatterns, mobile cells fail to respond to linear chemotactic gradients and Nucleus-Golgi polarity is not a universal characteristic of individually migrating cells. However, Nucleus-Golgi polarity can be induced by adding select features of the cancer environment back into the system. These findings highlight the utility of having the ability to add or subtract individual components from a well-defined multicomponent system.
Chapter 1: Introduction

Cell migration is a fundamental characteristic of cancer metastasis

Cancer metastasis accounts for 90% of cancer-related deaths (Siegel et al. 2013). During this process, cancer cells disperse from the original tumor and migrate throughout the body to form secondary cancer sites. In many cases, this migration depends on cells developing the ability to move. Epithelial to Mesenchymal Transition (EMT) is one way in which cells reacquire migratory capabilities. Therefore, EMT is thought to play an important role in cancer metastasis.

During EMT, epithelial cells transition from being stationary cells that are tightly bound to their neighbors to more motile and invasive cells. Rather than being a singular change, the EMT process involves a spectrum of effects that include altered morphology and protein expression leading to progressive loss of cell-cell contacts and increased motility. These changes can be prompted by a variety of different signaling pathways. Where cells end up on the EMT spectrum is a result of the cell type, the signaling pathway that initiated the transformation and the local environment (Lamouille et al. 2014).

Cancer cell migration occurs within a complex environment that has proven difficult to replicate in vitro

The tumor microenvironment (TMEN), has been shown to play a large role in cancer progression. Crucially for the study of metastasis, the TMEN is full of cell motility signals (Rodriguez & Schneider 2013). For example, solid tumors are harder than the surrounding tissues setting up a stiffness gradient that could promote durotaxis (Friedl &
Wolf 2010); gradients in the deposition of fibronectin and other matrix proteins provide haptotactic signals (Oudin et al. 2016); and local cells secrete a variety of soluble factors including CXCL12, EGF, and CSF1 which are all known promoters of chemotaxis (Mosadegh et al. 2008; Evanthia T Roussos, Condeelis, et al. 2011).

In addition to this multitude of overlapping, motility-inducing gradient fields, structures in the surrounding tissue and extracellular matrix (ECM) have features that may serve to focus and enhance motility (Rodriguez & Schneider 2013; Sidani et al. 2006). The passage of neutrophils or highly invasive cancer cells through the ECM leaves tracks of degraded matrix that can be followed by less invasion competent cells (Carey et al. 2013). Collagen fibers have been shown to form aligned bundles radiating outward from the tumor that could assist outward movement of cells (Provenzano et al. 2006). Additionally, pre-existing structures such as the lymph drainage system or the outside of blood vessels can form paths for cell migration (Pang et al. 2015).

_in vitro_ and patient studies have shown that these features, individually and together, play a large role in both the growth and dissemination of cancer cells (Desruisseau et al. 2006; Provenzano, Inman, Eliceiri, Trier, et al. 2008). The prevalence of tumor associated immune cells has also been shown to lead to lower survival rates (Grivennikov et al. 2010). Live cell imaging in mice has revealed a close spatial relationship between tumor associated immune cells and cancer cells. _Ex vivo_ studies revealed an EGF/CSF1 paracrine loop that allows the immune cells to lead the cancer cells from the primary tumor to a nearby blood vessel thus aiding in metastasis (Evanthia T Roussos, Goswami, et al. 2011). Recently, fibronectin gradients outside of

1-2
the tumor and toward blood vessels have been shown to be indicative of increased disease recurrence and the fibronectin gradients have themselves been shown to be crucial drivers of hepatotaxis when recreated *ex vivo* (Oudin et al. 2016).

These studies depend on three different levels of observation and experimentation. First, the importance of these biological systems is observed in the clinic. Next, confirmation and more detailed characterization are acquired from live animal studies. Final proof of mechanism relies on the use of a controlled, manufactured environment that recreates the essential aspects of the system while allowing use of existing high resolution biological tools for imaging and manipulation.

*Combining several tissue features in an ex vivo platform would allow the controlled reconstruction of in vivo environments*

One common characteristic of these environments that has been difficult to recreate is the alignment of the physical environmental features with the other graded aspects (Provenzano, Inman, Eliceiri, Knittel, et al. 2008). For example, the degree of collagen remodeling into aligned fiber bundles has been linked to poor patient prognosis (Conklin et al. 2011). Intra-vital imaging in mice has shown that tumor cells prefer to migrate along aligned collagen fiber bundles to escape the primary tumor (Sidani et al. 2006).

There is currently no accessible way to recreate topographical features of the microenvironment along with one or more of the gradients observed to co-exist *in vitro*. We seek to address this ongoing need for an ex-vivo platform that will permit a more
detailed study of the mechanisms involved in multi-parameter motility. Such a platform needs to combine surface topologies that shape and direct cell movement with soluble or adsorbed gradients. This combination will significantly aid the understanding of metastasis and other cell-environment interactions by recreating aspects of the complex features of the physiological environment while permitting cell behavior to be probed and analyzed in as much detail as possible.

Existing technologies can mimic single features of the cell environment

The tools necessary to start building combination microenvironments are already well established in two separate techniques; microcontact printing and microfluidics. Both techniques use polydimethylsiloxane (PDMS), a soft polymer with a silicon backbone (Théry 2010; Mcdonald et al. 2002). Microcontact printing relies on uniformly coating a 3D structured PDMS “stamp” with the desired molecule. The stamp is brought in contact with a surface and the molecule selectively transfers based on which parts of the structure contact the surface. This method allows fine spatial control of 2D surface features. The technique was originally demonstrated by the Whitesides group and relies on the surface being more accepting of the molecules than the stamp. Since then, many others have produced variations of this technique to build different surface chemistries, including thiol bonding to gold, hydrophobic adsorption, and surface erosion. Generally, these surfaces are uniformly coated with the adsorbtion/binding chemical with an added layer in the microcontact printed area. This poses a challenge for securely attaching a microfluidic device to the same surface. The most common, and
most secure, method to attach a microfluidic device to a glass surface requires treating the glass surface and the device with plasma. After plasma treatment, the two surfaces are placed in contact and the silicon backbone of the PDMS device covalently bonds to the silicon surface. The intermediate layers prevent covalent bonding of the device and the plasma treatment destroys the micropatterns. Our system (demonstrated in Chapter 2), overcomes these challenges and allows diverse micropatterns to be readily combined with microfluidic devices in a robust manner using no additional lab equipment.

*Environment influences cell polarity*

When an individual cell migrates away from its origin to a new environment, it does so either by forging its own path through the ECM or by following a favorable, pre-existing path. These paths can be quite varied. Normal, non-invasive, human mammary epithelial MCF10A cells have been observed to follow the channels generated by highly metastatic human mammary epithelial MDA-MB-231 cells as they invade through collagen gels (Carey et al. 2013). In the clinical setting, studies have highlighted the formation of aligned collagen fiber bundles radiating orthogonally from primary breast tumors (Provenzano et al. 2006). Cells migrating from the tumor were observed to prefer to follow the aligned fibers over navigating the more disordered normal collagen matrix. Similar to the aligned fiber bundles, migrating cells also encounter the natural ductwork of the body, such as the lymph system, and have been shown to utilized these features to facilitate movement (Pang et al. 2015). Despite the many different types of paths, there are some common aspects that they share. The paths tend to force the cell
into an elongated shape either through the alignment of the environment, in the case of fiber bundles, or by virtue of being a narrow channel and physically constricting the cell. In addition to elongation, the narrow environment also restricts movement. Although cells could conceivably invade the wall of the channel, the easiest path is along the channel. This restricts the cell to bi-directional movement along the channel while retaining an elongated morphology due to the narrowness of the channel.

It is well known that the quantitative efficacy of cell migration depends on a number of sub-cellular events that occur in separate regions. Such activities include membrane extension, actin polymerization, and Rac1 signaling at the front of the cell, and the release of membrane, force generation and RhoA signaling at the rear (Petrie et al. 2009). Environmentally induced elongation and restriction serve to create a more distinct and spatially separate front and rear of the cell.

**The position of the Golgi in relation to the nucleus establishes an asymmetry in the cell**

The establishment of front-rear polarity is thought to depend, at least in part, on the Golgi Position Relative to the Nucleus (GPRN). Several features of the Golgi standout as possible reasons for this role (Millarte & Farhan 2012). It is closely associated with the microtubule organizing center, MTOC. The initiation of movement has been shown to involve the MTOC/Golgi centering in the cell while the nucleus moves toward the rear (Hale et al. 2011). The Golgi is capable of nucleating its own microtubules which extend laterally across the Golgi stack but also toward the membrane where they may direct protein traffic or provide mechanical support (Zhu &
Kaverina 2013). The membrane nearest the Golgi receives higher vesicle traffic than the rest of the cell (Miller et al. 2009).

_Golgi Position Relative to the Nucleus may be involved in directional cell migration_

Although the orientation of the Golgi to the nucleus has long been considered relevant to cell migration, the exact nature of this relationship is unknown (Millarte & Farhan 2012). In 2d sheet migration and wound healing, disruption of GPRN leads to less effective wound closure. Disrupting Golgi position in other systems disrupts cell motility as well (Hurtado et al. 2011; Bershadsky & Futerman 1994; Deakin & Turner 2014; Bisel et al. 2008; Miller et al. 2009). Meanwhile, migration in an elongating environment appears to change GPRN compared to migration in 2d contexts. Pouthas et al. showed that in epithelial-like cells, the Golgi switches from being ahead of the nucleus to behind as the width of the migration path narrows (Pouthas et al. 2008). Doyle et al. observed that fibroblasts migrating on a fibrillar 2D surface had their Golgi positioned in the back of the cell between the nucleus and the trailing edge (Andrew D Doyle et al. 2009).

How GPRN is regulated by EMT is unknown and has potential implications for how front-rear polarity is achieved or maintained in cells that are evolving invasive capability. As both EMT progression and GPRN are sensitive to the environment, it is important to study the effects of EMT on cells and GPRN in the confined environments that cells will be migrating in.
Micropatterned lines mimic the constraints of migration paths found in vitro

Several methods already exist to replicated the elongating and restrictive characteristics of physiological migratory paths. Channels that confine cells are straightforward to build out of PDMS and numerous studies have been conducted in this system (Balzer et al. 2012; Pathak & Kumar 2012). However, the walls and roof of the channels, while more physiological dimensionally, also add complications to the study of migration. Cells have been shown to sniff out and follow chemotactic gradients created by the limited diffusion in narrow channels (Scherber et al. 2012) but such external directional biases would obscure the influence of elongation alone. Another technique, microcontact printing, allows the covalent linking of narrow protein lines on glass. Unprinted areas can be rendered non-adhesive by adsorbtion of PEG or similar molecules (Xia & Whitesides 1998). This combination of narrow adhesive lines separated by non-adhesive lanes allows the cell to be forced into an elongated morphology and imposes bi-directional choice, but still allows nutrients and signals to freely diffuse in a non-biasing manner around the cell. Thus, micropatterning provides a well understood and readily tunable platform to study the effects of elongation on cell migration.

Single cell studies in a controlled environment have the potential to expand our fundamental understanding of motility and enable new therapies

Much of the detailed Golgi function studies related to cell motility have been performed in sheet migration and wound healing assays. The microenvironment of
these cells is very different from that of single cells in that it contains cell-cell contacts as well as asymmetric forces and space availability. Here, we look at the Golgi positioning of single cells that are free to migrate in a symmetric space. This allows Golgi effects to be decoupled from cell-cell effects so that the connection between Golgi position and migration can be assessed more directly. Our aims measure the basic physical characteristics of Golgi repositioning. This provides a basis for future hypotheses of what Golgi positioning can possibly do and the time scales at which it can be done.

The discovery of novel cancer drugs requires a deeper understanding of how the drugs impact cell behavior in a tumor like environment. This system combines a complex yet tunable microenvironment, capable of single cell measurements, with a subcellular readout of a polarizing element. Understanding the extent to which this polarity marker responds to environmental perturbations and drives cell behavior will be a step towards developing a new type of ‘tumor mimetic’ assays and screening technologies.
Chapter 2: Integrating micropatterns and microfluidics to study the interplay of molecular and physical spatial cues

1. ABSTRACT

Cells perceive and respond to cues that provide spatial information about the microenvironment. This information can be conveyed by geometric physical constraints, such as those imposed by fibers, pores and channels, and by molecular gradients of extracellular ligands. Microcontact printing and microfluidic devices each provide effective platforms for studying cell response to physical geometric constraints and molecular gradients, respectively. In many in vitro scenarios, geometric and molecular cues work in concert, and in applications such as tissue engineering, both cues can be employed to manipulate spatial patterning and organization of cells and tissues. To enable investigation of the interplay between geometric cues and molecular gradients, we report here a facile method to incorporate any micropattern geometry within any microfluidic device. The method requires no specialized equipment beyond what microfluidics labs routinely use. It involves producing micropatterns on a silicon-based substrate (e.g., glass or PDMS), suitably protecting the micropatterns during plasma treatment, and subsequently aligning and bonding the substrate to PDMS microfluidic channels. We show that the method yields a micropatterned-microfluidic (MPMF) device capable of generating a gradient of soluble ligands above a substrate with micropatterned ligands. Patterns range from simple shapes (circles or squares), to more complex shapes, such as rings of tear-drops and an annulus. We demonstrate that non-transformed mammary epithelial MCF10A cells cultured in the MPMF device spread
and migrate while confined to the micropatterns, confirming that the protection scheme effectively shields the micropatterned proteins during plasma treatment. MPMF devices provide a versatile platform to investigate the synergistic role of physical and molecular cues in cellular spatial sensing, including the acquisition and maintenance of cell polarity.
2. INTRODUCTION

Spatial features of the cellular microenvironment play a significant role in regulating a wide range of cell behaviors, including migration, proliferation, cell death, and gene expression (Polyak et al. 2009). This has motivated the development of technologies to fabricate platforms in which critical spatial features of the microenvironment may be mimicked and tuned, while also being amenable to quantitative analysis and screening of the cellular response (Toetsch et al. 2009). Two broad categories of commonly-engineered spatial features involve molecular gradients and physical constraints (El-Ali et al. 2006; Théry 2010; Rodriguez & Schneider 2013).

Spatial gradients in molecular ligands constitute a significant class of cues that promote cell chemotaxis, proliferation, and cell fate decisions (Zheng et al. 2014; Lee et al. 2004; Evanthia T Roussos, Goswami, et al. 2011). Microfluidic devices provide a platform to present precise molecular gradients and study their effects on cell migration (Whitesides 2006; Keenan & Folch 2008). These include gradients of soluble (Shamloo et al. 2008) or surface-bound molecular species (Hsu et al. 2005). Soluble gradients can be established in the presence (Jeon et al. 2000) or absence (Haessler et al. 2009) of flow. Furthermore, the technology has matured to the point of combining multiple gradients to investigate synergistic effects (Kim et al. 2010).

In addition to molecular cues, the microenvironment contains structural and physical guidance cues that influence cell migration (Rodriguez & Schneider 2013), spreading (Kumar et al. 2011), polarization (Kandere-Grzybowska et al. 2010), death (Dike et al. 1999) and other responses (Théry 2010). These cues are defined by the
architecture of the extracellular matrix, ECM, such as fiber alignment, (Provenzano et al. 2006) pore size (Wolf et al. 2007) and luminal position (Nelson et al. 2005). A proven and effective strategy to study the role of physical constraints and local ECM geometry in regulating cell behaviors is the use of micron-scale adhesive patterns (Anderson & Hinds 2011). One relatively straightforward method to make such patterns involves microcontact printing (Xia & Whitesides 1998). Applying this technique, cell behaviors such as apoptosis (Chen 1997), migration (Kushiro & Asthagiri 2012; Kushiro et al. 2010; B. Chen et al. 2013; Jiang et al. 2005), protein expression (Li et al. 2008), proliferation (Dike et al. 1999), gene expression (Vartanian et al. 2010), and cell differentiation (McBeath et al. 2004) have been shown to be sensitive not only to gross features, such as micropattern area, but also detailed features, such as curvature and asymmetry, and relative positioning of micron-scale adhesive regions.

A physiologically significant challenge is to better understand how cells respond to combinations of molecular and physical guidance cues. This question is particularly relevant in an environment in which features, such as molecular gradients and physical spatial constraints, co-evolve during disease progression (Polyak et al. 2009). Incorporating micron-scale patterns into microfluidic devices would open the door to investigating how geometric cues and molecular gradients work together to regulate cell processes, such as migration. However, current methods to embed micropatterns within microfluidic devices compromise the range of micropattern shapes that can be studied.

One common technique is to first assemble the microfluidic device and then use the laminar flow characteristics of microfluidic devices to create separate fluid streams
in the main channel (Takayama et al. 1999). The ligands in each stream are deposited onto the substrate, and provided the flow rate is sufficiently high, inter-stream mixing by diffusion is limited and the borders between patterns are rendered with fidelity. While this approach allows multiple ligand mixtures to be patterned within a single microfluidic channel, the micropattern shapes are limited to geometries with edges parallel to the direction of laminar flow, such as rectangular stripes that extend along the full length of the microfluidic channel. Sharply-angled or fine-featured patterns cannot be produced. Even arrays of simple patterns, such as square or circular adhesive patches, that are commonly used to study the effect of adhesive geometry on cell behavior (Théry 2010) could not be made by flow-based methods.

Another method involves uniformly coating the glass substrate with the desired ligand and then using a PDMS stamp to protect regions to be micropatterned while plasma treatment is used to etch away unprotected regions. Following plasma treatment, the substrate and PDMS microfluidic channels are readily bonded. While this approach expands the set of micropattern shapes that can be incorporated in a microfluidic device, some shapes remain out of reach. This method requires a pathway for the plasma to reach the region to be etched and for the plasma reaction products to exit. These conditions are not met for micropatterns with fully enclosed, non-patterned regions in their interior such as an annulus. Micropatterns with interior gaps, such as centered and skewed ring-shaped annuli and square-shaped frames, have proven important in dissecting the dependence of cell proliferation (Chen 1997) and migration behaviors (Kushiro et al. 2010; Kushiro & Asthagiri 2012; B. Chen et al. 2013; Jiang et
al. 2005), respectively, on spatial geometric constraints. Furthermore, the walls of these enclosed regions will experience a pressure differential during the vacuum and repressurization stage of the plasma treatment process resulting in the potential deformation or displacement of the patterning features.

Other more intricate methods also exist such as filling a channel in a fully-assembled microfluidic device with protein and then selectively etching away undesired proteins or selectively cross-linking desired proteins by applying lasers or UV light through the PDMS channel roof (Anderson & Hinds 2011). However, these methods suffer from their own sets of drawbacks and compromises such as limited spatial resolution, complex chemistries, and additional equipment costs.

Here, we demonstrate a facile technique for incorporating micropatterns of any arbitrary shape into any microfluidic device. It combines common existing microcontact printing and microfluidic manufacturing methods and does not require specialized equipment, making the method widely accessible to labs currently working with microcontact printing and microfluidics.
3. MATERIALS AND METHODS

*Fabricating device and stamp molds*

We used SU-8 photoresist (MicroChem) on silicon wafers to generate both devices and stamps. Briefly, SU-8 50 (devices) or SU-8 2010 (stamps) (Microchem) was spin-coated onto 3-inch silicon test wafers (Silicon Sense). The coating was exposed to UV light (Quintel 4000) through a chrome/soda lime mask (Front Range PhotoMask). Non-cross-linked portions were etched away using SU-8 developer (Microchem) leaving 3D positive relief features (ridges) for the device and 3D negative features (wells) for the stamps. PDMS mixed with cross-linker (10:1 devices, 8:1 stamps) (Dow Corning, PDMS 184) was poured over the molds and cured for at least 2 hours at 80°C (fig. 2.1a).
Figure 2.1: Device fabrication overview.
(a) Basic Photolithography steps to make stamp and device molds. (b) Microcontact printing. (c) Device bonding over stamped patterns.

Device and stamp preparation

PDMS slabs containing the channels or stamp features were peeled off the silicon wafer and cut to size. Holes were punched in the slabs with channels using a biopsy punch (Miltex) and covered with scotch tape until the bonding step. Prior to stamping, stamps were placed ridge side up and covered in a mixture of PBS (Life
Technologies), 10 µg/ml fibronectin (Life Technologies) and 1.5 µg/ml Alexafluor – 594 (Life Technologies) overnight on a rocker at room temperature.

**Substrate preparation**

PDMS-coated coverslips were prepared by spinning 8:1 base/cross linker PDMS on 50 mm round cover glass (Wilkim Scientific) at 6000rpm on a spin coater (Laurell Technologies Corporation) for two minutes. The PDMS was cured at 80° C for at least 2 hours and stored at room temperature until use.

**Device and stamp alignment**

In typical microfluidic application, which we demonstrate here, the device has a rectangular experimental channel and the stamp is the same shape. This produces three degrees of freedom that must be addressed: the x-direction along the length of the channel, the y-direction along the width of the channel, and the angle of rotation between the patterns and the channel. Controlling all these variables presents a challenge because it is very difficult to see the microscopic features on the stamp and device.

Pattern to device alignment is the most important and most challenging step in the protocol. Only one attempt at alignment is possible since any contact between the plasma-treated PDMS device and PDMS-coated surface will initiate bonding. At the same time, this contact must happen within 10-30 seconds after plasma treatment to ensure a fully intact bond and prevent leakage. This means device bonding has to be a
quick and precise action. Below are several manufacturing and technique tips that assist with device to pattern alignment.

1) Make a wide, ~1mm, border around the stamp. This makes it easier to cut the stamp to a specific shape and ensure that the stamp features are aligned to the edge of the stamp. The alignment of the PDMS stamp must be the same as the alignment of features on the stamp.

2) Make the stamp bigger than the microfluidic channel in which the patterns are to be incorporated. This allows some flexibility in placement.

3) Make a mold for the PDMS protector. This ensures a consistent area is protected even when new protectors are used. The mold should be the same length as the channel, in the x-direction, but slightly wider in the y-direction. We use a protector that is 300 microns wider than the channel.

4) Placing a flashlight so that it shines along the counter top helps illuminate the features of the stamp and device. This is particularly useful when bonding the device because it highlights the edge of the main channel. It also assists in cutting stamps and punching holes accurately.

5) On the opposite side of the coverslip that is to be stamped, use a lab marker to draw a line that is the same size as the channel to create a “channel marker”. Ensure that the line is place such that the device can fit on the surface if the device channel is over the line. The stamp, the PDMS protector, and the channel of the device will all be aligned to this marker.
6) Working on top of a clean transparency sheet over the lab bench helps highlight the dark features, such as the marker line, without obscuring the lighter features on the PDMS.

When stamping (see below for more detail), the stamp is placed so that the channel marker is in the center of the stamp. This ensures that there is flexibility for the placement of the protector and device. Care is taken to ensure that the long axis of the stamp is not rotated with respect to the channel marker. Before plasma treatment, the PDMS protector is placed so that an edge of the channel marker is centered in the Y-direction. Lab markers typically create a line that is much bigger than a channel so the edge of the line is a better visual reference than the center of the line. Lifting and replacing the PDMS protector does not damage the stamped proteins. This allows multiple attempts to align the protector. After the protector is placed, briefly exposing the surface to the flame from a Bunsen burner will reduce the rate of plasma chamber contamination by preemptively removing some of the proteins. After plasma treatment, the protector is removed and the device channel is aligned to the same end and edge of the channel marker as the protector. Since only one attempt at bonding is possible, use of the techniques described above to aid alignment is crucial.

**Stamping**

Stamping was done in accordance with Tan et. al. (Tan et al. 2004) The PDMS-coated surface was plasma treated for 2 minutes on high (Harrick Plasma). Stamps were gently rinsed in DI water and blown dry with nitrogen (Airgas) and immediately placed face down on PDMS centered on the black line. Slight pressure was applied on
the top of the stamp to ensure full contact and the stamped surfaces were left to sit in
the dark for at least 2 hours (fig. 2.1b).

Device bonding to the patterned substrate

Device bonding was accomplished similar to regular device bonding except that
instead of a blank substrate, a stamped substrate with a PDMS pattern protector is used
(fig. 2.1c).

Stamps were removed from the cover glass. A rectangular sliver of PDMS
slightly larger than the device cell chamber was placed over the black line to protect the
newly stamped protein from plasma treatment. The cover glass and device were placed
in the plasma chamber and plasma treated for 25 seconds on high (Harrick Plasma).
The PDMS protector was removed, and the device was immediately bonded using the
black line as a visual cue (fig. 2.1c). After bonding, a solution of 0.2% Pluronic F-127
(wt/vol) (EMD Biosciences) in PBS was immediately added. The increased
hydrophilicity of the newly plasma treated PDMS channels helps prevent the formation
of bubbles when first injecting fluid in the device while the Pluronic blocks cell adhesion
in the non-patterned areas. After 10 minutes, the Pluronic solution was flushed out
using media, and cells were injected at the desired density.
4. RESULTS AND DISCUSSION

Fabrication method to incorporate micropatterns in a microfluidic device

To enable studies of the interplay between molecular gradients and physical geometric cues, we sought to develop a method to integrate micropatterned surfaces into microfluidic devices. A design objective was to be able to incorporate any micropattern shape of interest. These geometries include shapes, such as stars and triangles, which are impossible to make by standard flow-based patterning. In addition, we sought to incorporate micropatterns with interior gaps, such as an annulus, which would be difficult to fabricate using etching-based methods (Rhee et al. 2005).

Unlike flow-based methods that produce micropatterns after fully assembling the microfluidic device, our strategy was to prepare the micropatterned substrate and subsequently bond it to a PDMS microfluidic channels to combine both features in a single device. We use microcontact printing to produce micropatterns of the desired protein(s) on spin-coated PDMS substrates. We reasoned that because the substrate is not yet housed in a microfluidic device, it is fully accessible for the complete diversity of shapes permissible by classical microcontact printing. Separately and in parallel with the micropatterning, a section of PDMS containing channels for fluid flow and a chamber for cell culture is prepared.

As with micropatterning, there are multiple available methods to bond the microfluidic PDMS channels onto the micropatterned PDMS substrate including passive bonding, external clamps and PDMS surface modifications (Temiz et al. 2015). In order
to make our technique as broadly applicable as possible, we chose covalent bonding by plasma treatment which is the most common bonding method. In addition to being widely used, plasma treatment offers the strongest available bond, requires the fewest additional assembly steps, and requires no external equipment to maintain the bond (Wasay & Sameoto 2015). To ensure that the micropatterns are not damaged during plasma treatment, we cover the patterns with an unmodified sliver of PDMS, a method used to protect regions of substrates from plasma activation (Rhee et al. 2005; Hattori et al. 2011). Subsequently, the microfluidic PDMS channel is bonded irreversibly onto the micropatterned, plasma-activated substrate, yielding the micropatterned, microfluidic (MPMF) device. The bonding step is done with special attention to the alignment of micropatterns and the walls of the cell culture chamber in the microfluidic device (see Materials and Methods for details).

**Patterns and the gradient retain fidelity after bonding**

We first tested that our fabrication method yields fully functional MPMF devices that do not compromise either the physical geometric cues or the molecular gradient. To evaluate the micropatterns, protein solutions containing fibronectin doped with a small quantity (1µg/ml) of fluorescent Alexa-flour 594 tagged Bovine Serum Albumin (BSA-594) were used for microcontact stamping. Meanwhile, to generate and monitor the molecular gradient, we used solutions of Alexa-flour 488 tagged BSA (BSA-488) in a microfluidic gradient generator modeled on the original design from the Whitesides group (Jeon et al. 2000).
Examining the micropattern features after assembling the MPMF device confirmed that complex shapes were rendered with high fidelity (fig. 2.2a,b). A detailed look at the variety of shapes shows that hollow patterns, such as the annulus, are rendered with the same fidelity as the solid square next to it. Patterns with small gaps (square of tear drops), sharp corners (star), and dead ends (three sided square) are all feasible. These observations demonstrate that shielding the micropatterns with a PDMS sliver during plasma treatment protects the shapes from distortion. Thus, this method enables adhesive patterns of any shape amenable to classical micropatterning to be incorporated into the controlled fluid environment of a microfluidic device.

Meanwhile, visualizing the BSA-488 confirms the presence of the molecular gradient (fig. 2.2c). The top and bottom walls of the microfluidic chamber are indicated with arrows, with the arrowhead pointing in the direction of flow. These results demonstrate that the unshielded regions of the substrate receive adequate exposure to plasma treatment, activating the surface to form a seal tight enough to sustain fluid flow in the device.
Figure 2.2: Complex surface features combined with a microfluidic gradient generator.

(a) A field of complex micropatterns (BSA Alexafluor-594) post protection and plasma treatment. (b) A zoomed in image of one block of micropatterns showing that they are still intact after plasma treatment (inset from a). (c) A spatially defined soluble molecular cue (BSA Alexafluor-488) above diverse microcontact printed patterns (same patterns as 2a) with well-defined location and orientation. (e) (inset from d) Non-flow based patterns successfully aligned to the wall of a microfluidic chamber. (Yellow arrow indicates the edge of the chamber.)

We next examined the placement of the micropatterns in relation to the molecular gradient. Figure 2.2d shows a device in which the patterns are offset from the molecular gradient. The gradient, visualized by a green fluorophore, overlays the micropatterns in red. The groups of micropatterns are rotated in every direction showing that the shapes can be made independent of the orientation of flow or the device walls (yellow arrows).
Micropatterns are visible below the bottom wall but not above the top wall, revealing the mismatch between the protected region and the microfluidic chamber. Above the device, no patterns are visible due to the gap between pattern groups that places the next set of patterns outside of the image. Below and inside the device the micropatterns are completely intact. This example shows that even with a substantial mismatch the device is still useable and highlights how our design choices make this a viable technique.

The imperfect alignment is a result of the design tradeoff between technique accessibility and precision. We chose plasma bonding since it is the most common method and, except for passive bonding, the least elaborate. This requires rapid alignment during the bonding process. Alignment is done by visual surrogate since the micropatterns are not discernable by eye. This means that some mismatch is inevitable, especially along width of the channel which is the smallest dimension. The choice is between making the patterned area smaller or bigger than the chamber. In the first case, the pattern is always fully enclosed by the chamber but chamber space is wasted. In the second, the chamber is always full but the patterns extend beyond the walls.

We considered the second case to be the most experimentally desirable because the entire chamber is always accessible. It also requires the sturdiest device construction. As a result, we made the PDMS protector 300 µm larger than the chamber. At full mismatch, the upward force on the chamber roof from the fluid pressure has a 300 µm “lever” with which to pry at the bond between the PDMS channel and the substrate. Figure 2.2c shows that the PDMS wall fully blocks the flow of the
green fluorophore. This means that the device bonding is sufficiently strong in the plasma cleaned area to prevent fluid from leaking under the wall or breaking the bond.

**Patterns are aligned to the gradient**

Pattern placement influences cell placement since cells can only adhere to the patterned area. Cell placement in a device is crucial because the environment of a microfluidic channel is not uniform. Fluid shear stress and local concentration within the molecular gradient will vary with distance from the walls, both of which can have effects on cell behavior (Hsu et al. 2005; Li Jeon et al. 2002). Depending on the device chosen, the position of the cell along the length of the channel can determine the concentration of nutrients or secreted factors to which the cell is exposed (Kim et al. 2010).

In addition to the spatial coordinates of each pattern within the gradient, the rotational alignment of the pattern relative to the molecular gradient is an important parameter that regulates cell polarity and behaviors, such as directed cell migration. Thus, tuning the alignment of the micropattern shape with the axis of molecular gradient is essential to investigate the interaction between physical and molecular polarity cues.

The angle between micropatterns and a channel in the device is accurately aligned by eye using the techniques outlined in the Device and Stamp Alignment portion of the methods (fig. 2.2e). The speed and accuracy of alignment is central to the flexibility and general nature of this technique. Slower alignment would require a non-covalent bond between the glass surface and the PDMS device since the surface activation from the plasma treatment decays rapidly. This would require bonding
methods such as an external clamp to ensure that the device does not leak (Temiz et al. 2015). However, an external clamp significantly increases the foot print of the device and makes it more challenging to connect tubing to the device or image the main channel. Less accurate alignment would require a reversible method that allows for repeated attempts to place the device properly. This could be achieved through “passive bonding” which relies on Van Der Waals forces between the PDMS and glass surface to keep the device in place. This limits the flow rate in the device since internal pressures must be kept less than 5 psi (Mcdonald et al. 2002). Furthermore, connecting tubing to the device or bending tubing already in place can easily break the bond between the device and surface. In contrast, the covalent bond created by plasma treatment produces a foot print that is exactly the size of the device and glass coverslip and is the strongest bonding method available. As a result, we were able place a micropatterned, gradient device inside the covered, heated stage of a microscope and image live cells overnight.

**Stamped proteins retain functionality after plasma treatment and device bonding**

The use of a PDMS protector raises several concerns. Since the protector physically contacts the patterns this may cause the patterns to smear or the proteins may adhere to the protector and be removed with it. Additionally, PDMS is gas permeable so oxygen that is present throughout the PMDS may be converted to plasma during the plasma treatment process. During this process, the plasma chamber produces a radio frequency electric field oscillation specifically designed to generate
oxygen plasma. If oxygen plasma is generated in sufficient quantities near the surface, this plasma could interact with the protected proteins and damage them. To verify that the patterns retain biological functionality, MCF10A cells were seeded on lines and shapes. Cell behavior was observed for 14 hours. MCF10A cells repeatedly traversed the same stripe of fibronectin (fig. 2.3a) and conformed to the shape of the pattern (fig. 2.3b). Cell confinement to the boundaries of the micropatterns is consistent with cell behavior on patterns outside of microfluidic devices (Théry, Racine, et al. 2006; Andrew D Doyle et al. 2009) and indicates that the biological functionality of the fibronectin was not significantly perturbed.
Figure 2.3: Cells remain viable and confined to micropatterns after plasma treatment.

(a) An MCF10A cell repeatedly traversing a printed line of fibronectin in a device. (Yellow arrow indicates the edge of the microchannel.) (b) Contrast enhanced images of an MCF10A cell. Left, a microcontact printed pattern preserved during plasma treatment. Mid, Phase image of the cell spread to fill a fibronectin pattern in a device.

Alternate stamping methods can also be used

Since the initial demonstration of microcontact printing in the Whitesides lab, many more surface chemistries have developed. We have found that as long as the surface is capable of bonding to PDMS, our method allows the chemistry to be used. We have successfully used both liquid and vapor deposition of silanes which eliminates the need for a spinner (data not shown).
5. CONCLUSION

*Ex vivo* analogues of microenvironmental features have been a critical tool for researchers to explore the influence of specific microenvironmental characteristics such as gradients and geometric confinement. As the impact of individual features become apparent, there is a growing need to study those features in conjunction with one another.

We report a new method to combine the techniques of microcontact printing and microfluidics in a single device thus enhancing the ability to select and study surface and soluble environmental cues simultaneously. Importantly, this method requires no additional equipment beyond what is typically needed for microfluidic studies and utilizes materials familiar to any lab that uses PDMS for microfluidics or microcontact printing. Our technique enables any shape that can be micropatterned on a silicon based substrate, such as glass or PDMS, to be combined with any gradient generating microfluidic device. The gradients and features can be accurately oriented to each other enabling their interactions to be spatially tuned and studied. We have demonstrated the versatility and efficacy of this technique by combining a variety of complex shapes, such as an annulus, with a fluorescently imaged gradient. We culture non-transformed mammary epithelial MCF10A cells in the MPMF device and show that they remain confined to those shapes during migratory or static studies.

This combination of surface patterning and control of the fluid environment enables a wide range of biologically relevant studies. Any substrate controllable cell configuration such as shape, orientation, cytoskeletal configuration, or surface protein
attachment is able to be studied in the context of spatially defined molecular cues. This permits a select number of parameters from the physiological environment to be combined in a controllable fashion so that their effects on cell behavior can be studied.
Chapter 3: Role of molecular and geometric spatial cues in determining polarity of non-motile spread cells: the static case

1. ABSTRACT

Having developed a single platform in which spatial patterns in both molecular and physical cues could be presented simultaneously, we sought to investigate how spatial gradients in molecular cues would work together with geometric spatial constraints to regulate cell polarity. We focused first on cell polarity in a static context in which a cell is adhered and spread onto an adhesive substrate but cannot move out. To impose this static condition, we seeded cells on micropatterned shapes no bigger than 32 µm on a side. The micropatterns were surrounded by regions coated with Pluronic, which prevents protein adsorption and cell attachment. Micropatterns of different shapes were chosen to vary the symmetry of the cell shape by altering the physical and geometric constraints. In addition, pattern orientation to a gradient of soluble ligand, epidermal growth factor, was varied. Gradients were generated using a flow based, microfluidic device that created linear gradients along a rectangular chamber. We found that our control case with serum free media and no flow had an unexpected bias. Flow altered the bias slightly and EGF applied in either a uniform or linear gradient removed the bias. These findings suggest that an unknown paracrine signal induces a bias in MCF10A cells in serum free media and that local concentrations of cells could be used as gradient point sources in future experiments.
2. INTRODUCTION

Asymmetric, micropatterned shapes are commonly used to alter the polarization of cells (Théry 2010). The Whitesides group seeded cells on teardrops and showed that when the cells are released they migrate toward the broad end of the cell (Jiang et al. 2005) although this polarization was short lived. Other groups have shown that the internal organization of cells is also susceptible to micropatterned shapes. Micropatterned crossbow shapes encourage the cell to align the Nucleus-MTOC, NM, axis toward the curved front of the crossbow while symmetric squares held no observed NM bias (Théry, Racine, et al. 2006). Circles and triangles have been used to show the same effect for the microtubule organizing center and the nucleus (Hale et al. 2011).

These shapes were all chosen to duplicate the observed shape of cells in different migratory environments. By eliminating cell-cell signaling and pressure from adjacent cells the influence of shape alone can be investigated. Squares crudely duplicate the shape of a cell packed tightly in the middle of an intact epithelial sheet. A triangle, crossbow or teardrop mimics the shape of cells at the edge of a damaged epithelial sheet and solitary, unmoving cells in a dish tend to be circular. All these studies point to the idea that shape asymmetry is sufficient to impose a migratory direction on a cell.

Gradients of soluble, molecular factors have also been shown to bias cell migration in vitro (E T Roussos et al. 2011) and in microfluidic devices (Saadi et al. 2006). Multiple gradients have even been used to trap cells in a specific location (Meier
et al. 2011). To date, however, the influence of chemotactic gradients on NG or Nucleus-MTOC positioning have not yet been studied.

Our first goal was to establish the baseline Nucleus-MTOC, NM, polarity of MCF10A cells confined to a static shape. This would allow us to determine if shape alone was sufficient to induce bias in a breast cancer relevant cell line. Then, by applying an external, chemotactic gradient, the interplay between shape induced biases and gradient induced bias could be studied.
3. RESULTS

To determine the influence of cell shape on Nucleus-MTOC polarity, non-transformed human mammary epithelial (MCF10A) cells were seeded on 32 µm wide fibronectin micropatterns adsorbed to PDMS. After cells adhered to the micropatterns, non-adherent cells were rinsed off and exposed to serum free media overnight. Patterns were arrayed in groups containing one of each pattern as shown in figure 3.2a. After exposure, cells were rapidly fixed and stained to highlight the MTOC and the nucleus. Cells were then imaged and analyzed using a custom, semi-automated Matlab program.

The average Nuc-MTOC orientation of solitary, fully spread cells was analyzed as shown in figure 3.1. The separation distance between the nucleus and MTOC was included as a weighting factor in the analysis since that separation between the MTOC and nucleus has been shown to proceed migration in wound healing studies involving fibroblasts (Gomes et al. 2005). Angle of the NM axis with respect to the patterns symmetric axis was measured in the cases where a single axis was present. Otherwise, the symmetric axis of the large teardrop was used as the reference axis. Figure 3.2a summarizes the average NM direction for cells on patterns in an open dish.
Figure 3.1: Sample data from the star pattern.

Nucleus-MTOC angle to individual patterns is plotted in the right two figures. This angle weighted by the separation distance between the Nucleus and MTOC (top left and center) and plotted (bottom left and center).
Figure 3.2: The MTOC exhibits an unexpected orientation for cells cultured on micropatterned surfaces in the absence of a gradient in soluble factor.

Arrows and numbers indicate the average direction of an arrow drawn from the cell nucleus to the MTOC. N is the number of fully spread cells used to compute the average. The averages shown were deemed statistically significant if they had a p-value <0.05 by both Rao's and Rayleigh's tests for circular statistics. All cells were cultured in serum free media. A) (left) Averages from cells cultured in open dishes. B) (right) Averages from cells cultured in a microfluidic device with a flow rate of 100 nl/min flow. Green numbers are the averages new average orientation. Shapes are, from top to bottom, left to right: oval, angled line segment, line segment, small tear drop, triangle, X, T, star, plus, crossbow, circle, square, pentagon, large teardrop.

Not all of the shapes had a statistically significant average. This alone is not unexpected since some of the patterns, like the circle, should not induce a polarity, and the large tear drop was not analyzed since it was generally occupied by a group of cells due to its size. However, the true cause of the lack of significance was low numbers of single, fully spread cells. All of the shapes were designed to fit in a 32 µm wide square to avoid elongating one shape more than another. An unintentional side effect of this design choice is that different patterns have different adhesive areas. The square, on which cells tend to not fully spread, has an area of 1024 µm². The triangle, which has a
useable number of cells, has half that area, 512 µm². Thus, excessive surface area eliminated 6 of the 14 patterns from use.

The remaining patterns exhibit a mono-modal average when they would be expected to display at least a bimodal distribution based on symmetry. If cells on a line segment favor pointing off to one side, there is no pattern based reason that they should point to one side over the other. Likewise, the T, the triangle and the crossbow pattern should either have a single average toward the front or have two equally common “modes” toward either side of the center. Based on previous work by Thery et al, we expected these patterns to show a strong NG bias toward the front of the pattern. Instead, they show a single mode to one side of a bilaterally symmetric pattern. The fact that all the distributions exhibit a single mode indicates that something is overriding whatever bias the pattern might be imposing.

The appearance of a bias in the control is intriguing as it hints at a currently unidentified source of NG bias. As discussed above, since the bias does not follow the pattern symmetry we can conclude that the patterns are not the source. We can also discard a whole chamber bias due to the positioning of the patterns groups (discussed in the next section). When we look at the four “2D” patterns (the triangle, T, crossbow and star) we see that the NG axis points to the right, so the bias is either toward the right or away from the left. To the right of each of these patterns is the large tear drop. As noted earlier, this pattern is large enough to accommodate multiple cells. A full tear drop holds between 10 and 12 MCF10A cells so the most obvious explanation is that
these cells are secreting a paracrine factor that is influencing the orientation of nearby cells.

If this paracrine factor exists, it does not affect the line segments or the small teardrop which make up the 1D patterns. There are three possible explanations for this. The 1D patterns are further away than the 2D patterns so the concentration of paracrine factor could be too low. Alternatively, the geometry of the 1D pattern could override the factor or the cells could be influenced by another source from the cells on the large teardrop of a nearby pattern group. The last is the least likely as the large teardrop within the pattern group is marginally closer (~ 32 microns). The first two reasons cannot be de-convoluted in this system but it is unusual that the line segments also have mono-modal distributions. This suggests that something is still influencing their NG polarization beyond the pattern. To further examine potential causes of the unexpected bias, patterns were placed in a microfluidic chamber where flow and exogenous gradients could be applied to the cells.

_Chamber wide biases, such as flow, were controlled for by rotating pattern groups_

Although the microfluidic device adds additional experimental options, it also requires additional controls. Flow is necessary to create the gradient, provide the cells with nutrients and counter media evaporation through the walls of the device. A high enough flow rate could also be used to remove factors secreted by the cells thus potentially eliminating the influence of the unknown biasing factor. However, shear
stress from fluid flow has also been shown to elongate and orient cells thus providing a potential additional source of NG bias (Lee 2004).

We tested for flow induced or other whole chamber biases by altering the position of pattern groups in the chamber. The pattern groups (a rectangle containing one of each pattern as shown in figure 3.2a) are rotated 90° from each other to sample 4 possible orientations and the patterned area of the dish was always set square to the microscope. This means that a full dish bias would result in a distribution with 4 modes rather than one. Since flow is orthogonal to the gradient direction we can distinguish between a flow induce bias and a gradient induced bias.

During the flow experiments, media was supplied to the cells at a flow rate of 100 nl/min. The chamber volume is 1.2 microliters so this effectively emptied the chamber every 12 minutes. In the absence of EGF, the monomodal biases returned although they were shifted slightly from their positions in static media (fig. 3.2b). This shows that the flow was not enough to remove the paracrine signal and, also, not fast enough to create its own bias.

Exogeneous signaling factors were added to override or obscure the unknown biasing factor

The next series of experiments sought to determine the source or nature of the NG polarizing signal while keeping the patterns the same. This choice was heavily influenced by manufacturing challenges. Specifically, shrinking the pattern size, while keeping the same relative proportions, would have required us to be able to maintain
pattern fidelity on feature sizes below 5 microns. Although smaller sizes have been demonstrated with microcontact printing, our lab has had little success below that barrier and alternative methods, such as e-beam lithography, are much more expensive.

One possible source of bias is EGF being secreted by the cluster of cells on the large teardrop, which is a known chemoattractant for MCF10A cells (Palmieri et al. 2006). Radial diffusion would set up a gradient which may be sufficient to cause the single cells on nearby patterns to orient themselves toward the cluster. This gradient could be obscured by adding a large amount of EGF to the media, removing the paracrine bias and allow the pattern bias to dominate.

In order to test the idea that an EGF gradient was causing the observed NG bias, EGF was added to serum free media at a concentration of 4nM which is about 4 times higher than the $K_d$ of the EGF-EGFR bond. This was expected to fill many of the EGF receptors and make it harder for a cell to sense a subtle gradient.

In the presence of exogenous EGF, cells had no NM bias. This means that the endogenous signal was overridden and the pattern bias, if present, was also overridden. Since EGF is also a motility factor (Mosadegh et al. 2008) as well as a chemoattractant, the cells may simply be too motile to be influenced by the patterns. A lower concentration of EGF, 2nM, was used to see if motility could be reduced while still obscuring the gradient. However, cells still exhibited no bias at the new lower concentration.
Rather than continue to search for what could be a very narrow concentration band where the patterns imposed a bias but the unknown paracrine factor was swamped by added EGF, an exogenous gradient of EGF was applied to see if it imposed a NM bias. Two gradients, 0.1-2 nM and 0.0-4 nM EGF, were supplied using the device described in Chapter 1. A gradient-induced bias should be indicated by 4 modes in the distribution, each mode corresponding to one of the four orientations of the pattern groups. In the case of a resurgence of the endogenous bias, made possible by the low concentrations of EGF at the bottom of the gradient, a mono-modal distribution would be observed. Instead, cells continued to exhibit no bias.

At this point, no pattern bias had been observed and experimental numbers were low due to the patterns being too big for the cells. To correct these deficiencies a new, larger, cell type was used, NRK52E cells. These are rat kidney epithelial cells and are much larger than 10As. Early attempts showed them spreading fully on even the largest patterns. Unfortunately, they shriveled and died shortly after fully spreading. The whole cycle of spreading, shrinking and dying only took about 2-3 hours which made it challenging to apply a gradient for sufficient time to test for a bias. Eventually, it was concluded that the cells were removing the adsorbed fibronectin and dying when they no longer had sufficient adhesive area (Chen et al. 1997). This motivated the development of a covalent linking chemistry that could be combined with a microfluidic device and enable longer term studies of cell confined to micropatterns.

Once the proper covalent chemistry was found, the gradient generating device was developed enough that it could be used to apply gradients to motile cells instead of
static cells. Since NM polarity was interesting due to its presumed influence on cell
motility it was decided that studying the influence of gradients directly on motility was
more likely to yield interesting results. As a result, static patterns were traded for line
patterns that spanned the width of the experimental chamber. These experiments will
be discussed in depth in chapter 4.
4. DISCUSSION AND CONCLUSION

Table 3.1: Table of experiments demonstrating the removal of the Nuc-MTOC bias in the presence of EGF

<table>
<thead>
<tr>
<th>Flow condition</th>
<th>Media</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>static</td>
<td>SFM</td>
<td>bias</td>
</tr>
<tr>
<td></td>
<td>4 nM EGF</td>
<td>No bias</td>
</tr>
<tr>
<td></td>
<td>2 nM EGF</td>
<td>No bias</td>
</tr>
<tr>
<td>100 nl/min</td>
<td>SFM</td>
<td>bias</td>
</tr>
<tr>
<td></td>
<td>0.1-2 nM EGF</td>
<td>No bias</td>
</tr>
<tr>
<td></td>
<td>2 nM EGF</td>
<td>No bias</td>
</tr>
</tbody>
</table>

The Nucleus-MTOC, NM, orientation of stationary, untransformed, human, mammary epithelial cells was looked at in response to four experimental conditions (Table 3.1). Every cell occupied a micropatterned area. Some micropatterns had a shape designed to bias the cytoskeletal organization of the cell and impose a NM orientation. This base orientation was perturbed by three media conditions; serum free media (SFM), SFM with uniform concentrations of epidermal growth factor (EGF), or gradients of EGF. Two major results were observed during these experiments. In serum free media, cells on 2D patterns exhibit a bias such that an arrow drawn from the Nucleus to the MTOC points toward the large teardrop in the pattern group. The addition of EGF, either uniform or in a gradient, removes this bias. This behavior has several possible explanations.

MCF10A cells are known to cluster when seeded in a standard tissue culture dish. This clustering is more aggressive in serum free media (Pope et al. 2008). The
simplest explanation is EGF secretion by the clusters of cells on the tear drops. Radial diffusion from a “point” source sets up a gradient of EGF, a known chemoattractant for MCF10A cells, that encourages cells to pull together. If this is the case, then the addition of exogenous EGF may be overriding the signal and removing the bias.

All the same, the removal of the bias when EGF is added is odd. One of the ways in which EGF has been shown to increase cell motility, is by reducing the number of focal adhesions that cells form. Other studies (Théry, Pépin, et al. 2006) have demonstrated that when cells are seeded on micropatterns, focal adhesions tend form and cluster at the sharp corners of the patterns. Actin stress fibers radiating from these clustered focal adhesions alter the stress distribution of the cytoskeleton and have been shown to influence the positioning of organelles such as the nucleus. So, in the absence of EGF, cells should be firmly adhered to the micropattern and that relationship should dominate. When EGF is added, the structural influence of the micropattern should be diminished with the decrease in focal adhesions and soluble biases should play a bigger role. Instead, the opposite happens.

Another potential explanation exists. Some cells have been shown to possess an innate chirality. When seeded on large circular patterns, the whole group of cells will migrate in a clockwise direction (Segerer et al. 2015). In other cases, when cells are seeded on an annulus, the outer cells will migrate in one direction and the inner cells will migrate in the counter direction. Since most of the cells with an unexplained bias are triangular, it is possible that the pattern symmetry tells them they are in a particular position within a sheet of cells and should begin migrating to their right. This explanation
provides another reason why cells would retain their bias regardless of how the pattern
groups are rotated, although the biased star pattern remains an anomaly.

Unfortunately, these two alternate explanations make it impossible to say if the
pattern or the soluble gradient is the dominant factor in the biases seen here. It is also
unclear if the biasing factor is innate to a single cell or a result of signaling from nearby
cells. What is clear, is that NM biases are present in stationary, single cells and that
they are responsive to environmental factors.
Chapter 4: Role of molecular and geometric spatial cues in determining migratory bias: the motile case

1. ABSTRACT

Cell migration involves the acquisition and maintenance of front-rear cell polarity. At the front edge, cells extend lamellipodia; meanwhile, contractile forces preferentially detach the cell body at the rear, resulting in net forward motion. Cells achieve directed migration by defining their front-rear axis in relation to cues that provide spatial information. Soluble cues presented in a spatial gradient can serve as a chemotactic cue, directing front-rear polarization toward higher or lower concentration. Meanwhile, geometric constraints have been shown to affect cell polarity. Both of these motility features have clinically relevant impacts. Chemotaxis plays an important role in breast cancer metastasis, and therefore, we focused on a breast cancer cell line that has been reported to exhibit chemotaxis in vitro in microfluidics devices. Aligned fiber bundles have also been observed during in vitro studies of breast cancer and ex vivo studies have shown that micropatterns can control cell polarity. Although both features have been observed in breast cancer, how they combine to control and direct cell motility has not yet been tested. Here, we used our newly developed platform to investigate chemotaxis in the context of narrow adhesive stripes that emulate fiber-like microenvironment found in tumors. We find that linear gradients do not induce chemotaxis either with or without a micropatterned guide.
2. INTRODUCTION

Placing line patterns in a chemotactic gradient allows us to combine the \textit{ex vivo} analogs of two important \textit{in vitro} features; gradients (Shields et al. 2007) and aligned collagen fibers (Provenzano et al. 2006). When the lines point up the gradient, these two cues are expected to work together to enhance chemotaxis. Cell on lines tend to be more elongated than their planar counterparts (Andrew D. Doyle et al. 2009), this should extend the cell body up the gradient and enhance the differences in front-rear signaling thereby increasing the cells ability to sense the gradient.

Furthermore, we expect the persistence of cell migration to increase. Confinement to the line should heighten the energetic cost of turning since the cell must turn completely around to change direction rather than taking a slight angle. In other words, cell polarity should be maintained for longer. Some preliminary evidence for this exists. Our group has shown that cells seeded on square arrays of teardrops will continuously rotate in the same direction around the square (Kushiro et al. 2010). When the distance between the head and the tail of the teardrop was lengthened by several cell lengths, cells continued to migrate without changing direction. This suggests that the polarity necessary for the cell movement across the gap was retained by the cells moving along micropatterned lines (Kushiro & Asthagiri 2012).

In chapter 3 we looked at ways to create a directional bias in the Nucleus-MTOC axis of stationary cells. In Chapter 4, that goal was broadened to measure any directional bias that influenced how a cell actually moved. This was accomplished by placing individual cells on covalently bonded 10 µm wide collagen lines or on a plain 2D
stamped collagen surface. Three chemotactic agents were used: EGF, CXCL12 and 10% fetal bovine serum. Net displacement, persistence, speed and velocity were measured using manual tracking and custom MATLAB code to determine if cells were biased toward or away from the gradient. Despite the use of both EGF and CXCL12 as chemoattractants, and a wide range of gradient slopes, no displacement bias was observed. This suggests that a critical aspect of the gradient microenvironment was missing.
3. METHODS

Experimental parameters were selected to meet microfluidic culturing requirements while avoiding new biases

Experiments were designed to reduce the influence of all factors except the gradient and the lines. The base conditions used serum free media (SFM) on 10 µm wide, covalently bonded, collagen lines with a chamber flow rate of 100 nl/min. Alexaflour 488 bonded to bovine serum albumin, BSA, was also added to the media with the high concentration of chemoattractant to allow visualization of the gradient. This allowed us to check the gradient for each experiment to ensure that it was established and oriented as expected.

Using serum free media eliminated the unknown constituents in the fetal bovine serum, FBS, as well as the variations in constituent levels between batches of FBS. One of these constituents is likely EGF as FBS contains large amounts of growth factors so including FBS would have altered the concentration of EGF which is one of our experimental factors.

Collagen was chosen for the surface ligand because cells were able to adhere and spread quickly on collagen, ~25 min. Quick spreading is necessary because flow cannot be supplied to the device before the cells are adhered. If left too long, >1hr, cells begin to die. The volume of media in the experimental chamber is quite low, 1.2 µL, and the deaths appear to be due to the depletion of the small number of nutrients available to the cells since they die more quickly in SFM. Due to this, cells were seeded in normal growth media and the chamber was flushed with SFM prior to the experiment. The other
concern is that the chambers are made of PDMS which is permeable to water vapor. Even in a humidified environment such as an incubator, cells begin dying in the chamber around the 2hr mark due to media evaporation and bubble formation in the experimental chamber. Thus, rapid cell adhesion to the micropatterned collagen lines permits us to work around two common hazards of working with microfluidic devices.

When selecting flow rate, the low end of the flow spectrum was chosen to avoid the influence of shear forces on cell polarization. A minimum of two inlets are needed to form a linear gradient so the chamber flow rate is the combination of all the inlet flow rates. In this case, a syringe flow rate of 50 nl/min into two inlets gave a chamber flow rate of 100 nl/min. An inlet flow rate of 50 nl/min was chosen as it was the lowest consistent flow rate that the syringe pumps could deliver. At lower flow rates the pumps would occasionally stall during an overnight experiment.

A number of metrics were devised to look for biases in cell movement. These metrics were dependent on the geometry the cells were confined to so they are divided into metrics for cells moving on lines or on a flat surface.
Figure 4.1: Sample data set from one run in experiment 4. A) Top is a uniform concentration. B) Bottom has a gradient.
Metrics used to analyze polarized cell movement along 1D stripes

Net displacement:

The core metric is net displacement (shown in the upper left hand corner of figure 4.1a). This metric measured the functional result of a migratory bias without needing to track a cell for the entire duration of movement. If cells are biased to move in a particular direction, on average, cells should displace more in that direction than in others. In this system, a positive displacement indicates that the cells moved toward the high concentration of the gradient. For uniform concentrations, the positive direction is where the gradient would be if the gradient chamber were superimposed over the uniform chamber. Analyzing the uniform and gradient chamber using the same axis allowed us to filter the data for any device wide biases that influenced movement, although no such biases were ever observed.

Average cell velocity: Average cell velocity was measured to see if there was a difference in cell speed depending on if cells were moving up or down the gradient. This metric used the same orientation as net displacement, so positive was up gradient and negative was down gradient.

Average speed: Average speed, a non-directional metric, was measured to see if there were changes in how cells moved that could not be captured by directionality. For example, cells could move faster in response to a gradient compared to a uniform concentration, even if they do not move faster in a particular direction.
**Persistence:** Persistence was measured to see if cells moved more consistently in one direction over the other. Positive numbers indicate persistent movement toward the high side of the gradient.

**Location:** The final two metrics were combination metrics and were used to look at net displacement as a function of initial position in the chamber. These metrics were intended to identify biases in cell motion based on where they were in the chamber. The bottom center figure focuses on location along the length of the channel. Position along the length of the channel is important because the gradient flattens out the further down the channel a cell is. Ligands in a given bolus of fluid have more time to diffuse as the bolus moves down the channel so the high concentration gets slightly lower and the low concentration gets slightly higher. Had differences been observed between migration at the start of the chamber and the end, this would have told us what gradient slope the cells prefer to migrate along and could be modified by changing the speed of the flow.

The graph at the bottom right figure 4.1 looks at displacement along the height of the channel. Channel height dictates the average ligand concentration that the cell detects so this could identify a preferred concentration the cells migrate toward instead of simply migrating up or down the gradient.

*The experimental system introduces one unavoidable bias*

The only channel bias observed was in net displacement along the height of the channel (fig. 4.1a bottom right). Cells at the top of the channel are more likely to move down and cells at the bottom are more likely to move up. This is due to the fact that
cells are only analyzed until they collide with something such as another cell or the channel wall. This means that cells near a channel wall can only have large net displacements away from that wall. As long as the initial seeding is uniform, these biases should offset and still allow directional biases to be observed.
**Figure 4.2: Sample data set from 2D migration**

*Motility metrics in the absence of micropatterns*

Cells on a 2D surface can move freely in any direction along that surface. This requires different measures of persistence and directionality from cells confined to 1D lines. Since 2D surfaces are the usual surfaces for people to conduct gradient based chemotactic studies.

**Angle:** A line was drawn between the starting point of the cell and the end-point of the cell. The angle between that line and the fastest path up the gradient was measured in order to determine if a cell was generally moving up or down the gradient.
**Directedness**: This measures how close a cell moved to a straight line. The total path length of the cell is divided by the net displacement. A value of 1 means the cell moved in a completely straight line.

**Displacement**: This is the straight-line distance between the cells starting-point and end-point.

**Path length**: This is the total distance the cell travelled.

**Empirical cumulative distribution function, CDF**: This is a way of measuring how close a data is to a specific type of distribution. In this case, our data was compared to a normal distribution on the assumption that the displacements of unbiased cells would fit a normal distribution.
4. RESULTS

Table 4.1: Table of chemotaxis experiments using MDA-MB-231 cells.

All gradient studies were paired with a uniform chamber at the high concentration of the gradient. All net population displacements were near zero indicating that no migratory bias was present.

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Ligand</th>
<th>Flow Rate (nl/min)</th>
<th>Surface</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0-0.5</td>
<td>EGF</td>
<td>100</td>
<td>10 µm collagen lines</td>
<td>SFM</td>
</tr>
<tr>
<td>0.1-1</td>
<td>EGF</td>
<td>100</td>
<td>10 µm collagen lines</td>
<td>SFM</td>
</tr>
<tr>
<td>0-20</td>
<td>EGF</td>
<td>100</td>
<td>10 µm collagen lines</td>
<td>SFM</td>
</tr>
<tr>
<td>0-1</td>
<td>EGF</td>
<td>100</td>
<td>10 µm collagen lines</td>
<td>SFM + 0.5% FBS</td>
</tr>
<tr>
<td>0-10</td>
<td>EGF</td>
<td>100</td>
<td>2D adsorbed collagen</td>
<td>SFM + 0.5% FBS</td>
</tr>
<tr>
<td>1-10</td>
<td>EGF</td>
<td>100</td>
<td>2D adsorbed collagen</td>
<td>SFM + 0.5% FBS</td>
</tr>
<tr>
<td>0-1</td>
<td>EGF</td>
<td>100</td>
<td>2D adsorbed collagen</td>
<td>SFM + 0.5% FBS</td>
</tr>
<tr>
<td>0-1000</td>
<td>CXCL12</td>
<td>100</td>
<td>2D adsorbed collagen</td>
<td>SFM + 0.5% FBS</td>
</tr>
<tr>
<td>0-1000</td>
<td>CXCL12</td>
<td>200</td>
<td>2D adsorbed collagen</td>
<td>SFM + 0.5% FBS</td>
</tr>
<tr>
<td>0-1,1000,10%</td>
<td>EGF, CXCL12, FBS</td>
<td>200</td>
<td>2D adsorbed collagen</td>
<td>SFM + 0.5% FBS</td>
</tr>
</tbody>
</table>

To see how chemotactic gradients influenced the migration of cells on lines, highly invasive human mammary epithelial breast cancer, MDA-MB-231, cells were seeded in a microfluidic device on 10 µm wide collagen lines that were covalently bonded to a glass surface. Each device had two chambers, separated by a 300 µm wide wall, that shared a common outlet. This allowed cells to be simultaneously seeded.
in both chambers and for both chambers to share patterns made by the same stamp. One chamber served as the experimental chamber and had the gradient, the other chamber was a control at a uniform concentration equal to the highest concentration in the gradient chamber. All chambers were supplied media using the same syringe pump. Table 4.1 shows the combination of ligands, surfaces, flow rates, media and gradient concentrations used for these experiments.

**Starting alignment - Linear gradients with lines pointing directly up the gradient**

Initial experiments in SFM were conducted using EGF gradients of 0.0-0.5, 0.1-1 and 0-20 ng/ml. These concentrations were intended to target EGF-EGFR $K_d$ ranges of $<0.5$, $\sim 0.5$ and $>0.5$. Low $K_d$ was targeted because it is not uncommon for downstream signaling cascades to saturate at low receptor occupancy levels. If this saturation does occur in the case of EGFR signaling, variations in high concentrations of EGF would not be discernable by the cell. When cells failed to respond to low concentrations we used a higher concentration that would cover the range from 0 to 50% receptor occupancy. That also did not work so a $K_d$ high range, high steepness gradient was used. None of these ranges showed a bias in displacement, persistence or velocity.

Very little cell movement was observed in the first experiments so progressively steeper gradients, 0.1-1 and 0-20 ng/ml, of EGF were used. This did not increase cell movement and the cells did not demonstrate a preference for moving to one side of the chamber over the other. In order to increase movement and give cells an increased ability to explore their environment, 0.5% FBS was added to the SFM media. This
concentration is much lower than the concentration in normal MDA-MB-231 media which has 10% FBS. The goal was to add enough signaling factors to encourage movement but few enough factors to swamp the gradient signals. The added FBS was sufficient to roughly triple cell speed from 24 μm/hr to 60 μm/hr so all future experiments used this media.

The first experiment with substantial movement was experiment 4 with 0-1 ng/ml EGF, 100 nl/min flow in SFM + 0.5% FBS on 10 μm wide collagen lines. Sample data sets for the uniform and gradient chambers are shown in figure 4.3.

**Linear gradients on a 2D surface to remove the motility restrictions imposed by lines**

Due to the lack of bias, it was reasoned that the cause could be the micropatterned lines interfering with cell gradient response. It is possible that cells need to track back and forth in a gradient to sense the direction and the restriction to a 1D line prevented this behavior. To give cells this freedom of movement, we seeded them on uniform stamped collagen and placed them in the same gradient as before. However, we saw no motility bias with the change in geometric constraint.

A steeper gradient, 0-10 ng/ml, of EGF was tried since steeper gradients were used more often in the literature (Wang et al. 2004). Again no response was observed so the low end of the gradient was increased. Uniform increases in EGF concentration up to 2 ng/ml have been shown to increase motility (Mosadegh et al. 2008) so the low end was increased to see if cells only respond to a gradient if the base level of EGF is
high enough. No response was observed so a new chemoattractant was investigated to see if a change in diffusion rate and binding kinetics would inspire a different gradient response.

CXCL12, also referred to as SDF-1α, is another commonly used chemoattractant for MDA-MB-231 cells. The initial gradient used was 0-1 µg/ml based on the gradients reported in literature (Mosadegh et al. 2008; Fernandis et al. 2004; Lee et al. 2004). No chemotactic response was observed.

The only operational parameter that had not been changed at this point was flow rate. It was possible that if the flow rate of the media was too low, cells could consume enough of the ligand around them that they could no longer sense the gradient. Alternatively, a way to change the steepness of the gradient without changing ligand concentration is to change flow rate. For a given concentration, a low flow rate would have the flattest slope since the ligand would have the most time to diffuse. To see if increasing the steepness of the gradient and the availability of ligand, we doubled the flow rate to 200 nl/min. This did not work either, so a final experiment was conceived.

All of the gradients that had been applied to MDA-MB-231 cells were combined. Some evidence of synergistic effects between EGF and CXCL12 had been reported (Mosadegh et al. 2008) and 10% FBS has a lot of unknown factors that could also work synergistically. At the very least, combining ligands is a more efficient way of testing various conditions as long as none of them act counter to the others. A concentration of 0-100 ng/ml of EGF was used which was the steepest gradient reported by other labs
and 0-1 µg/ml of CXCL12 and 10% FBS were chosen as typical chemotactic concentrations. Again, no migration biases were observed.

Due to the lack of success imposing a polarity with a gradient, we turned to measuring the natural polarity of cells freely moving on micropatterned lines.
5. DISCUSSION AND CONCLUSION

Highly metastatic cancer cells were confined to narrow lines and exposed to two different chemotactic gradients. These conditions should approximate two major attributes of the tumor microenvironment. The micropatterned lines are analogous to the aligned collagen fibers that have been observed radiating outward from tumor edges and the gradient should emulate the soluble signaling environment.

The biggest question to come from these experiments is: why did none of the gradients bias cell migration? To answer this question, the literature was re-examined to identify differences between our assay and the reported assays showing a chemotactic response.

One of the most common assays takes place in a Boyden chamber. In this assay, fixed quantities of cells are seeded on one side of a porous membrane and the experimental media is placed on the other. Cells are given time to migrate through the membrane and then counted. If more cells migrate in the experimental media rather than the control, the ligand in the experimental media is considered a chemoattractant for that cell line.

This poses several challenges when compared to our assay. In a Boyden chamber, cells are only able to migrate in a single direction. The ligand could repel some members of the cell line and attract others. In our assay, the two displacements would offset each other and no net movement would be observed. In a Boyden chamber, only the chemotactic response would be noticed. Similarly, cells could simply
become more mobile. The mobility inducing ligand would appear to be a chemoattractant in a Boyden chamber because more cells would cross the membrane. In a bidirectional assay, there would be no bias because cells could migrate both toward or away from the ligand.

We observed a general difference between our assay and the common literature assays; none of the literature assays present truly linear gradients. Ligands are generally presented to the cell either through a porous membrane (R. Chen et al. 2013), diffusion from a pipet tip (Bailly et al. 2000), signaling from a nearby cell (Evanthia T Roussos, Goswami, et al. 2011) or through narrow, non-permeable channels (Scherber et al. 2012). In the first three cases, the ligand is diffusing from a point source, a hole in the membrane, the tip of a pipet or an individual cell. In the fourth case, the body of the cell fills much of the channel. Intake or binding of the ligand will remove it from the area around the cell. Since the walls are not porous, and the cell takes up most of the channel, ligand can diffuse to one side of the cell much faster than the other which sets up a concentration differential.

Using this insight, it is possible to design a microfluidic device that creates non-linear gradients within the plane where the cells migrate. Due to the laminar flow characteristics of all microfluidic devices, ligands move through the different fluid layers by diffusion rather than convective mixing. These layers follow the walls of the fluidic device. This is true even when the walls turn. So, we could create an experimental chamber where the walls snake back and forth and the gradient would follow them. If
the turn radius were tight enough, cells would sense a point source rather than a wavy linear gradient.

Overall, these experiments show that gradient steepness is not the only relevant parameter when using gradients to influence the migration of cells. Furthermore, although using micropatterned lines elongates the cells and allows them to sample more of the gradient than they normally would, this alone is not sufficient to induce chemotaxis in an open linear gradient.
Chapter 5: EMT and a fiber-like microenvironment bias MTOC positioning and increase migratory persistence

1. ABSTRACT

A combination of TGF-β treatment and high aspect-ratio micropatterns were used to examine the joint effects of EMT and confinement on the migration and MTOC positioning of individual MCF-10A mammary epithelial cells. We find that normal MCF10A cells have little cell-to-cell variation in either speed or persistence. Furthermore, the MTOC appears in the front or rear equally often, and neither position has an advantage in speed or stability. In contrast, the population of cells that have undergone TGF-β treatment exhibit, on average, increased speed, persistence and a preference for locating the MTOC in the rear. We find that the position and stability of the MTOC correlates with improved migration in single cells migrating in a fiber-like environment.
2. INTRODUCTION

Cells change their environment and at the same time, experience changes that other cells make to their environment. This is particularly true in the cancer microenvironment where normal cells are adjacent to a growing tumor and experience tumor related environmental changes without being cancerous themselves (Polyak et al. 2009). This leads to the question of whether the tumor causes the environment or whether the environment causes the tumor.

We sought to address this question in the context of breast cancer by selectively combining analogs of two features common to that tumor microenvironment; collagen lines and EMT. The relevance of both has been discussed previously (see intro and Chapter 4). Treatment with TGF-β was chosen as the means to induce EMT.

Clinical studies in humans have shown that TGF-β levels can serve as an indicator of cancer patient survival probability. Increased levels of TGF-β correlate with higher recurrence rates of cancer and lower survival rates (Desruisseau et al. 2006). Thus, TGF-β is a physiologically relevant inducer of EMT that can also be used to study this phenomenon in vitro (Zavadil & Böttinger 2005). In culture, it has been observed to promote EMT in a number of epithelial cell lines (Xu et al. 2009) including MCF-10A cells (Pang et al. 2015). TGF-β is produced by several cell types in the tumor microenvironment including by some metastatic breast cancer cells such as MDA-MB-231s. The inactive form of TGF-β is secreted by cells and sequestered on the cell membrane and surrounding ECM. After cleavage from its outer hydrophilic packaging, its active form is a 25 kDa homodimeric protein with two identical 112 amino acid chains.
linked by a disulfide bond. It binds to the TGF-βR2 receptor which recruits the TGF-βR1 receptor. This triggers signaling through several pathways. In the canonical pathway, SMAD localizes to the nucleus and up-regulates Snail. Increased Snail down-regulates E-cadherin which results in weakened cell-cell contacts thereby promoting EMT (Pickup et al. 2013).

Previous chapters looked at ways to induce polarity in cells. Chapter 3 looked at NG polarity in stationary cells while Chapter 4 looked for migratory bias in motile cells. In this chapter, we sought to connect NG polarity with migratory biases in single cells. Unexpectedly, these NG biases are unconnected to migratory biases in normal MCF10A cells. A connection is acquired when cells are treated with TGF-β. Migratory direction becomes mildly linked to Golgi position and migratory efficacy becomes strongly linked to the stability of the Golgi position.
3. RESULTS

In order to better understand how individual cells migrate in the tumor microenvironment, we looked at the migration of non-transformed human mammary epithelial cells confined to lines while monitoring the position of the nucleus and Golgi. Untreated or TGF-β-treated MCF-10A cells were seeded on 10 μm-wide micropatterned collagen lines. Treated cells were cultured in MCF10A growth media with an added 20 ng/ml of TGF-β for 12 days to induce EMT prior to experiments. The same concentration of TGF-β was added to the media during experiments. Micropatterned lines were created by covalently bonding collagen to a glass bottomed dish. Non-patterned portions were rendered non-adhesive by adsorption of Pluronic F127. Nucleus and Golgi position were labeled by expressing GFP fused to histone 2B and RFP fused to GM130, respectively. Positions were recorded concurrently by confocal, fluorescence, time-lapse microscopy over a duration of 14 h with Z-stacks covering the height of the nucleus taken every 2.5 min. The migration tracks were quantified by projecting the Z-stacks into a 2D plane where the centroids were automatically detected using a custom Matlab algorithm. There were 49 normal cells over 3 experiments and 61 TGF-β treated cells over 6 experiments.

**Speed and persistence were measured directly**

There are multiple methods in the literature to determine the speed and persistence of migrating cells. The simplest method is net displacement divided by total distance traveled and speed is total distance divided by total time. This method is commonly used but breaks down in a 1D context since it gives a high persistence to a
cell that moves very little but does not change direction and a low persistence to a cell that moves very far but changes direction and ends near the starting point. The gold standard method is to compute the mean square displacement, MSD, and simultaneously fit speed and persistence to the MSD equation (Meyer et al. 2012). However, this method is known to overestimate speed and underestimate persistence. Other methods also exist that more accurately measure these parameters (Gorelik & Gautreau 2014). However, unlike in traditional 2D and 3D migration studies, our cells are completely confined to linear motion and imaged frequently so speed and persistence can be directly measured without having to account for the possibility that the cell path curved between imaging frames.

Cell speed was determined for every frame using the displacement of the nuclear centroid between the previous and current frame and the time between frames (2.5 min). Persistence was defined as the distance the nucleus moved before stopping or changing direction. Averaging this produces the reported persistence of the cell and gives us a direct measurement of persistence rather than a derived one.

**EMT increases the speed and persistence of cells in a fiber-like microenvironment**

We measured the effects of EMT for cells migrating in a confined environment. Cells that had undergone EMT migrated faster and more persistently than their normal counterparts (Fig. 5.1). Normal cells moved at an average speed of 25.2 μm/hr. TGF-β treatment caused cells speed to double up to 51.6 μm/hr. Normal cells had an average persistence of 10.4 μm compared to 38.7 μm for EMT cells. These measurements show
that the EMT cell population was 4X more persistent while being only 2X faster than the normal cell population indicating that the combination of EMT and confining microenvironment have a greater effect on persistence than speed. This offers some insight into how EMT improves migration and what mechanisms are affected by TGF-β treatment. Increased persistence is likely caused by maintaining a particular polarized orientation for longer by enhancing the stability of the actin and microtubule networks as well as local concentrations of activated RhoA, Rac1, PI3K and other polarizing molecules. Increased speed, on the other hand, has to come from speeding up the grip, contract, release cycle of cell motility. Although both these two pathways are impacted by signals that bias cell polarity, different downstream mechanisms likely drive increases in performance.

Figure 5.1: TGF-β treatment enhances average population speed and persistence for cells migrating on lines. Treated cells are 2x faster (left) and 4x more persistent (right) than untreated cells.
After observing the differential effects of EMT on speed and persistence, we wanted to see how the two were linked in a fiber-like environment. Since our observations are direct measurements, rather than fitting to a MSD model, we can examine whether or not an increase in speed is linked to an increase in persistence by plotting the measured speed and persistence of each individual cell (fig. 5.2). We find that normal cells behave very similarly to each other and cluster together in the lower left hand portion of the plot with the majority of cells having speeds less than 38 µm/hr and persistence less than 19 µm. In contrast, almost half of the TGF-β treated cells have a speed or persistence greater than these limits.

![Graphs showing normal and TGF-β treated cells speed vs. persistence](image)

**Figure 5.2: Individual cells exhibit three different responses to TGF-β treatment.**

Non-responsive cells have the same average speed and persistence as untreated cells (bottom left section). Partial responders only have increased speed (bottom right). Full responders have increase speed and persistence (top right).

Although increased speed and persistence appear coupled in the population measurements (fig. 5.1), they are not very closely coupled in individual cells (fig. 5.2). A number of TGF-β treated cells have increased speed but their persistence is the same
as that of normal cells. At the same time some of the most persistent cells have only a minor increase in speed. Overall, it appears that the TGF-β treated cells fall into three groups based on their migratory response. Cells in the lower left hand corner have the same speed and persistence as normal cells so they were designated non-responders. The cause for the lack of response is unknown but could be a result of low numbers of TGF-β receptors or an insensitivity to TGF-β signaling among other mechanisms. These cells were binned based on their response being no higher than one standard deviation, SD, above the average response of the untreated cells. The next group of cells are located along the bottom of the figure. These cells have increased speed but their persistence is within one SD of the normal population response. They were called partial responders since only one aspect of their motility was enhanced. The final group is in the upper right corner of figure 5.2 and they have speed and persistence more than one SD above the normal cell population. Since both aspects of their migratory response are enhanced, they were designated full responders.

Given the variety of individual responses, we sought to understand what separated the different response groups and the discrepancy between the population and individual response in our study. To do this, it was necessary to have a readout that could be applied to both the population and individual cell level. We decided to look at Golgi position because it has a well-established influence on group cell migration (e.g. wound healing). At the same time, Golgi position can be identified for each individual cell for every frame of a video. This allows us to correlate Golgi position with migration properties for a range of scales including specific segments of motion, the entire length
of a single cell’s migration or between groups of cells. This provides readouts at the motion segment, individual cell, or population level.

**Line migration requires non-traditional binning of the Golgi position**

The first challenge was to determine how to bin the Golgi location and how to orient the bins. Golgi position studies typically choose bins based on a simplified geometry of the cell, an assumed central position of the nucleus, and the direction toward free space. A square cell on the edge of a sheet of square cells has a 90° window from the center of the cell toward the area the cell could freely migrate into (fig. 5.3). This results in 4 bins of 90° each. If the Golgi centroid is located in the bin oriented toward the sheet edge, the Golgi is said to be on the leading edge of the cell. Similarly, if the cell is approximated as a hexagon, a cell on the edge has a 120° window toward free space.
Figure 5.3: Strategies for binning Golgi position vary based on system geometry.
The binning strategies in wound healing assays are based on simplified cell shapes and migration toward free space. A dual bin strategy makes fewer assumptions about cell shape or direction and captures all possible movements for single cell migration on a line.

Although these are the two most common binning strategies, they do not fit the case of a cell migrating on a line. The free space in a line is quite limited and the nucleus position highly variable (Sengupta et al. 2013) so we chose to base our binning on asymmetry. We took into account the nuclear centroid, the Golgi centroid and the fact that the cell could only move in two directions. An off-center Golgi creates a structural asymmetry that is biased toward one side of the cell and one direction on the line. This gave two bins of 180° where the center of each bin pointed in different directions along the line. Bins were then oriented so that the leading edge was defined by the direction of nuclear motion. This gave rise to a variable Golgi orientation metric that is determined by the actual motion of the cell rather than the geometric constraints imposed by the position in sheet of cells. We called this metric Golgi Position Relative to...
the *N*ucleus, GPRN, where, in each image, cells have a GPRN state and this state can continue over multiple images.

To quantify the GPRN states of each cell, the positions Golgi and nucleus centroids were determined from fluorescence time-lapse images acquired at 2.5-min intervals. At each time point $t$, the orientation of the Golgi with respect to the nucleus was compared to the direction of migration. The direction of migration at a particular time point $t$ was identified from the shift in the position of the nuclear centroid from frame $t-1$ to $t$. We defined three GPRN state. In the ahead state (A), the Golgi is positioned in front of the nucleus relative to the direction of cell migration. The behind state (B) occurs when the Golgi is behind the nucleus relative to the direction of migration. The third unknown state (U) are instances that could not be identified as either the A or B state. The U state typically occurs either when the Golgi is undetectable or when the cell does not move, making the direction of migration inapplicable. After quantifying the GPRN state of the cell at each time point, a string of GPRN states, such as ‘BBBAAAUBBAABBBBB…UBB’, delineates the changes in the Golgi position relative to the nucleus at 2.5-min intervals as the cell migrates from its start point to its final location. In this example, the ahead state is measured five times in two continuous states with durations of 7.5 and 5 minutes, respectively.

*TGF-β treatment alters the default positioning of the Golgi*

GPRN states were measured for untreated and TGF-β treated cells to ask how TGF-β treatment alters the normal balance of GPRN states. We find that for untreated cells the Golgi splits time evenly between the ahead and behind states at 47.0% and
47.8%, respectively. The remaining 11.7% of the time, the Golgi is undetectable or the cell is not moving (fig. 5.4). The fact that there is a 50/50 chance of the Golgi being located ahead or behind the nucleus shows that MCF10A cells do not have a preferred Golgi position when migrating. This was surprising since several other studies involving individual cells migrating on micropatterned lines had cells with a clear preference in Golgi position (Pouthas et al. 2008; Andrew D. Doyle et al. 2009). These finding show that Golgi position, in single cells migrating in a confining environment, is not inherently linked to polarization. Furthermore, a particular Golgi position is not necessarily indicative of migration direction. Here, in a binary system, attempts to predict migratory direction based on Golgi position would fail 50% of the time for non-treated cells.

In contrast, after TGF-β treatment, cells acquire a clear preference for locating the Golgi in the back of the cell while migrating. The time spent in the behind state increases to 57.8% and time in the ahead state decreases to 34.8%. Although each shift is minor, together they mean that the Golgi is found in the behind state almost twice as much as it is in the ahead state. This means that the cells now tend to migrate in the direction opposite Golgi position. This contrasts with the widely reported Golgi forward bias seen in sheet migration during wound healing assays. The change in Golgi positioning between untreated and TGF-β treated cells suggests that role of Golgi position in migration may change as cells undergo EMT. To test this idea, we looked at how the migration properties of cells changed with Golgi position.
Figure 5.4: The TGF-β treated population positions the Golgi behind the nucleus more often.

Treated cells (left) spend twice as much time in the behind state as the ahead state. Untreated cells (right) spend the same amount of time in either state.

Golgi position correlates with a relative performance difference in cell migration.

The migration paths of individual cells were broken into segments of motion where the GPRN state did not change between imaging frames. The cell speed during each segment and the duration of each segment was calculated. Next, the segments were grouped by GPRN, and the average speed and duration of the behind and ahead motion segments for each cell was found. Ratios were formed of the average behind/ahead, B/A, segments for speed, duration and the total time cells spend in the two states. If these ratios are greater than 1 then, on average, that individual cell property performed better when the cell Golgi was located behind the nucleus.
Figure 5.5: Individual cells with enhanced migration exhibit differences between Golgi states.

The differences between the ahead and behinds states in each cell was examined as a B/A ratio and the ratio mean was plotted. Groups and Golgi states were compared using pairwise t-tests. Normal and Non-responder cells and their A and B states are indistinguishable from each other (p > 0.1). Partial and Full Responders are distinct from the other groups and have A and B states that are distinguishable (p < 0.05) but they are not distinct from each other. In responsive cells, the behind state of a cell tends to have a higher value than the ahead state. The exception to this is speed which is the same in all states and all groups.

Untreated cells have response ratios near one for all three properties (fig. 5.5) and the differences between the ahead and behind states are indistinguishable by a t-test. This shows that, in addition to cells spending the same amount of time in each state, cell speed and state duration are not influenced by Golgi position. Since the migratory characteristics are the same regardless of Golgi position, the mechanism for
Golgi participation in migration must work equally well in the front or back of untreated cells.

Alternatively, this suggests that the Golgi is not involved in migration. Arguments supporting a role for the Golgi in migration are based on the fact that the position and orientation of the Golgi is inherently biased toward one side of the cell due to its structure and the fact that it is offset from the nucleus (Millarte & Farhan 2012). Combined with observations of biased protein secretion from the Golgi during wound healing (Yadav et al. 2009), the Golgi appears to create a fundamental asymmetry in the cell. However, our data show that this inherent asymmetry plays no role in the migration of untreated cells.

This lack of Golgi influence extends to TGF-β treated cells that fall into the non-responder category (fig. 5.5). Cells in the normal and non-responder groups are indistinguishable from each other and the ahead and behind states of the non-responder group are also indistinguishable (p > 0.05). So, in addition to having the same speed and persistence as normal cells, the non-responsive cells also position their Golgi in the same way as normal cells.

Cells from the partial response and full response groups spend more time with the Golgi behind the nucleus (p < 0.05). These two groups are not statistically distinguishable from each other but are distinguishable from the normal and non-responsive groups. Cells with greater performance have the Golgi positioned in the back of the cell more than 86% of the time (fig. 5.5 yellow bars) as opposed to less than 66% of the time for non-responsive cells. These results show that positioning the Golgi
in the back of the cell is not just a general trait of TGF-β treated cells but is a trait specific to the subset of TGF-β treated cells that migrate more effectively. This suggests that placing the Golgi in the back of the cell is somehow linked to enhanced migration. We next sought to determine which aspect of migration was enhanced.

Speed ratios across all groups have a value of 1, showing that speed is not enhanced by changing Golgi position (fig. 5.5, blue bars). In turn, this indicates that the mechanism by which TGF-β increases cell speed is not directly mediated by Golgi position. An alternate way to increase average cell speed is to decrease the amount of time the cell is stationary. However, we found that both treated and un-treated cells spend less than 1% of their time being stationary, so neither a change in Golgi position nor a change in stationary time is sufficient to explain the speed increase associated with TGF-β treatment.

Since speed was unaffected by Golgi position, we looked at how state duration was affected. In this case, changing Golgi position does alter how long a GPRN state lasts in certain cells. On average, the behind state lasts more than 4 times longer than the ahead state for the high performing group. In non-responsive cells, the ahead and behind states last for the same amount of time (fig. 5.5 green bars). Once again, a change in how the Golgi is positioned is specifically linked to the cells with higher migratory performance.

Both high performing groups have longer duration of Behind states compared to the Ahead states and spend a greater percent of their time in the behind state. Taken together this means that the greater time in the behind state is achieved by increasing
the stability of the behind state relative to the ahead state rather than increasing the number of behind states or decreasing the number of ahead states. Since this is a three-state system, time out the ahead state is not necessarily time in the behind state. Equal duration behind and ahead states could be separated by unknown states and the greater time in the behind state could have been due to increase sampling frequency. This is particularly true in the case of TGF-β treated cells where the unknown state makes up 19.5% of the states. This distinction is important since sampling frequency and stability are likely modified through different molecular pathways.

Given that differential relative stability between the ahead and behind GPRN states correlate well with improved migration, it is important to see how this relative stability was achieved since it is possible that the ahead state becomes less stable rather than the behind state becoming more stable. To uncover how differential stability was achieved we plotted average state duration against migration performance (fig. 5.6). Once again, untreated cells and non-responsive cells were indistinguishable from each other and the ahead and behind state durations were indistinguishable. However, as migration performance goes up, state duration gets longer. Non-responders have a behind state duration of 6 min, partial responder’s behind state lasts for 12 min and full responders last for 34 min. All pair-wise t-tests for the behind state had p-values <0.05. This shows that as behind state stability goes up, migratory performance also goes up.

Ahead state stability seems to mirror this trend although only the partial responder and full responder groups are statistically different. Since both the ahead and
behind Golgi states become more stable, it is clear that the high B/A duration differential is achieved by improving the behind state stability more effectively than the ahead state.

Figure 5.6: Golgi states last longer in groups with more effective migration.
State duration was averaged across each performance group. The stability of both states increases as the cells become more motile. The behind state stability increases faster than ahead state stability. Behind state stability doubles then triples as response goes up. Normal and Non-responder groups are indistinguishable (p > 0.1). Ahead state Partial and Full responder groups are distinct. All behind state response groups are distinct (p < 0.05). Error bars are standard error.

These findings show that, for single cell migration in a fiber-like environment, Golgi stability is the relevant metric that separates highly migratory cells from regular cells. Increased stability is seen regardless of Golgi position but is more pronounced when the Golgi is behind the nucleus. Thus, the role the Golgi plays in migration efficacy and cell polarization is very different from what is observed in 2D sheet
migration. The preferred position of the Golgi is opposite of that seen sheet migration studies and position itself plays a less substantial role. Instead, the ability of a cell to maintain Golgi position is the distinguishing characteristic between slow, non-persistent migration and fast, persistent migration.
4. DISCUSSION AND CONCLUSION

Enhanced cell motility is a fundamental aspect of cancer metastasis which is an end-of-life event in many patients. As cancer progresses toward this stage, tumor cells and their surroundings undergo many changes. Understanding how these changes work together to enable cell migration is crucial to delaying or preventing metastasis. Here we have looked at Golgi positioning in human mammary epithelial cells after TGF-β treatment during migration on narrow collagen lines. This allowed us to examine a common polarity indicator in a cancer relevant cell line while simultaneously exposing the cells to a potent inducer of EMT and a common topographical feature of breast cancer.

In addition to being physiologically relevant, studying single cells confined to lines provides several advantages over the more traditional wound healing assays. In the line system, the cell can freely change direction so any movements observed are due to internal cell forces rather than external pressure from adjacent cells. This means each cell is analyzed individually. At the same time, the movement is linear and direction is binary. This simplifies analysis and, along with rapid imaging, allows us to decouple speed and persistence since they can be measured directly rather than derived from a model. Thus, Golgi association with direction, speed and persistence can be looked at in discrete time segments, individual cells or groups of cells.

We find that single cells in confined migration behave very differently from cells migrating as sheets in wound healing assays. This is important because mechanistic studies to understand the role of the Golgi in migration have almost exclusively relied on
wound healing as a model system. In wound healing assays, the default state of the Golgi is ahead of the nucleus toward the leading edge of the cell. When this orientation is disrupted, cells move less effectively and the wound takes longer to close. In contrast, during single cell, confined migration, the Golgi changes position frequently, every 7.5 minutes on average, and is found ahead and behind the nucleus equally often. Furthermore, cell speeds and state durations are the same in the ahead or behind state so the behavior of the cell is unchanged by Golgi position. This even distribution of positioning and performance demonstrates that, in normal MCF10A cells, there is no default position of the Golgi and no performance gain associated with a particular Golgi position. Thus, a lack of bias is normal in the single cell system and abnormal in the sheet migration system.

A Golgi bias and altered migration are acquired when cells are treated with TGF-β and undergo EMT. This allows us to start to understand how Golgi position influences migration. The first thing we see is that a Golgi bias and enhanced migration go together. The subset of TGF-β cells with average migration characteristics also have an unbiased Golgi. Highly migratory cells acquire a bias in Golgi relative positional stability where the Golgi is more stable in the back of the cell than the front. At the same time, the Golgi becomes more stable on an absolute scale such that behind state duration can be used to distinguish between each migratory subgroup. This demonstrates that enhanced migration and, thus, invasiveness are linked to increase Golgi stability. These findings have several important real world impacts.
The first impact is that Golgi stability is a desirable therapeutic target. Normal cells show little need for a highly stable Golgi, and invasive cells require stability. Thus, finding an appropriate molecular target, such as an upregulated microtubule stabilizing protein, would allow the disruption of cellular programming in cancerous cells while having minimal impact on normal cells. Such a dichotomy of response is highly favored as it allows a potent impact on cancer while minimally perturbing the function of normal cells. Additionally, we have now established a platform to study this cellular phenotype, to screen for appropriate stability disrupting drugs, and to further explore the mechanisms by which Golgi behavior influences migration.

The second impact is that increased Golgi stability is likely an unintended side effect of current cancer treatments. Some common cancer drugs, such as the taxols, function by increasing microtubule stability. This inhibits the mechanisms that allow a cell to divide thereby reducing excessive reproduction which is a hallmark of cancer. However, given the tight link between Golgi position and the position of the microtubule organizing center, MTOC, in mammalian cells, more stable microtubules likely means a more stable Golgi. If increased stability also causes increased migratory performance, this implies that while cancer drugs may impede one function of cancer, such as mitosis, they may inadvertently aid another, such as metastases.

These two impacts highlight the benefit of studying cellular perturbations in the context of the microenvironmental changes that occur alongside them. Past mechanistic studies looking for ways in which Golgi position could influence migration have focused on a Golgi that is positioned ahead of the nucleus and have not considered stability as a
metric. Our results show that elucidating the mechanisms by which localizing the Golgi behind the nucleus enhances migration may be more important for understanding the behavior of metastatic cells and could yield novel cancer therapies.
Chapter 6: Conclusions

Cell motility is a complex and essential process. It plays a role in wound healing, development, immune response and many other normal physiological functions. It also plays a role in physiological malfunctions such as cancer metastasis. Because of this dual role, cell motility must be understood as a contextual event rather than a modular characteristic that is easily ported between functions. A major part of the cell motility context is the changing physical environment in which the cell migrates and the diverse signals it receives from its surroundings.

A continuous motility assay is needed to understand the role of Golgi positioning in migration

The goal of this work was to try to understand how cells parse and combine multiple, external directional signals into a single migratory direction. This requires an improvement on the typical motility assay. The most common motility assays are monodirectional, end-point assays such as Boyden chambers and wound healing assays. In Boyden chambers, a fixed number cells are seeded on porous substrates. One side of the substrate has an experimental factor and the other does not. After the cells have had a chance to migrate, the number of cells that have migrated through the membrane are counted. Increases in cell numbers are taken to be proof that the experimental factor is a chemoattractant. The challenge with this interpretation is that cell movement is only measured in a single direction. If cells are simply more motile, they will also cross the membrane more often so a chemo-kinetic agent can be confused with a chemo-attractive agent.
Wound healing assays have a similar problem with separating out confounding influences. These assays are conducted by scratching a gap in a confluent monolayer of cells. The speed at which the cell layer closes the gap is considered indicative of how well the cells migrate. The challenge here is that some motility factors, such as EGF, are also proliferative factors. Therefore, wound closing may be due cells being pushed forward by an increasing number of cells behind them, rather than an intentional movement to heal the wound. This means that cell behavior is only measured by one metric and yet multiple, inseparable factors can influence that metric.

In order to understand cell motility in response to multiple external factors the opposite condition is necessary. The cell must be capable of multiple actions in response to a select and controllable number of inputs. This kind of study requires three primary components; spatial control of at least two independent directional signals, a means of evaluating migratory choice and a cell type relevant to the environment being investigated.

These components needed to be combined in a way that allowed wide-view, high-magnification, long-duration imaging of cells. Motility is an inherently time-based process so cells need to be imaged over a sufficiently long period to understand how snap shots of motion combine to form behavioral tendencies. At the same time, the cell needs to be imaged in sufficient detail that the mechanics of motility can be examined to form an understanding of how cellular decisions are made. On top of that, since cells are not staying in the same place, so it is necessary to image the places where cells might go in order to capture the times when they do go there.
Due to these imaging constraints, a planar migration assay was chosen and a device capable of combining micropatterned surfaces and soluble molecular gradients was developed. This reduced the possible combination of orientations that the two motility signals could take and simplified tracking since cells could not go up or down. Constraining movement to a plane also allowed much less space to be imaged so images could be taken more frequently, at higher resolution and over a wider area.

The specific features of the device were based on the metastatic environment observed in breast cancer. This was due to the large social impact of breast cancer and the availability of physiological data. This physiological data suggested that collagen fibers, increased TGF-β, and chemotactic gradients play a large role in breast cancer metastasis. The two motility cues were micropatterns to constrain or orient the movement direction, and chemotactic gradients to inspire movement in a particular direction. The cells used were human mammary epithelial cells, either untransformed or metastatic.

**Core Hypothesis: Golgi positioning dictates cell direction and is controllable by external cues**

The initial hypothesis of this work was that Golgi Position Relative to the Nucleus, GPRN, dictates cells direction and external motility cues dictated GPRN. The first part of that hypothesis was disproven by the results of chapter 5 when it was shown that cell direction and GPRN are not linked in untreated MCF10A cells. The biases observed in chapter 3 seemed to suggest that motility cues could dictate GPRN, since the GPRN of static cells was aligned in a manner consistent with the clustering behavior of MCF10A...
cells. Unfortunately, chapter 4 demonstrated that known chemoattractants do not create the expected migration biases in an open, linear gradient. This new information allows us to refine the circumstances in which the hypothesis is relevant and suggests alternate methods test it.

The unexpected results of chapter 3 can be used to overcome the challenges encountered in chapter 4 by providing a new method for gradient generation. In chapter 4, we struggled to find a gradient that cells would respond to. In chapter 3, we appeared to have inadvertently created a gradient that biased cells. A redesigned set of micropatterns, such as a large central circle with small, cell-sized (~500 µm²) circles around it, would allow us to confirm that a large cluster of MCF10A cells does secrete a GPRN biasing gradient. Replacing the smaller circles with lines oriented toward, or at angles to, the central cluster would allow us to test the hypothesis that the biasing gradient is a chemotactic gradient by measuring how cells move along the lines toward or away from the central cluster.

This path forward becomes a little more complicated when the results of chapter 5 are mixed in. In chapter 5, unmodified MCF10A cells in growth media had no preferred GPRN state when migrating on lines. In chapter 3, unmodified MCF10A cells in serum free media exhibited a bias which we interpret as the GPRN being in the ahead state since that is consistent with MCF10A clustering behavior. This bias, however, goes away when EGF, a constituent of growth media, is added. Based on more the results of Chapter 5, we can reacquire the bias by pretreating the cells with TGF-β. However, after TGF-β treatment, cells preferred to position their Golgi in the
behind state. So, the biases in unmodified cells on static shapes in SFM, appear to be the opposite of the biases in TGF-β modified cells in growth media.

The opposite positioning of the Golgi may simply be due to a change in the geometric constraints of the cells. Cells on the static patterns are not as elongated as cells on the lines. Pouthas et al demonstrated that shrinking the width of line patterns could flip the preferred positioning of the Golgi from ahead on wide patterns to behind on narrow patterns and this may be what is happening between chapter 3 and chapter 5. On the other hand, the GPRN bias may be the same, in which case, cells on static patterns are scattering instead of clumping and the unknown paracrine is a chemorepellant rather than a chemoattractant.

The impact of Golgi positioning is dependent on environmental factors that can be controlled

Previous work looking at the role of Golgi positioning has largely been conducted in wound healing assays which are group migration, end-point studies. In these studies, the Golgi is consistently positioned ahead of the Nucleus toward the leading edge of the cell (Millarte & Farhan 2012). When this positioning is disrupted, the wound heals more slowly. These results have led to the impression that Golgi positioning is both a constant and a necessity for directed migration.

The studies conducted in this thesis focused on the role of Golgi positioning in single cell migration. They have shown that, in single, stationary cells, Golgi positioning is biased by unknown factors and can be disrupted by adding EGF. Since EGF is a
motility factor, cells treated with it should be more motile and therefore have more consistent Golgi positioning. The fact that this is not the case, suggests that the link between motility and Golgi position may not be as fixed in single cells as it is in cell clusters. Further experiments made this link even more tenuous.

When the Golgi is continuously tracked, it becomes clear that Golgi position frequently changes relative to the direction of cellular motion. This happens on the time scale of minutes, demonstrating the highly active nature of this characteristic and further eroding the idea that consistent Golgi positioning is the default migratory state. In the single cell studies conducted here, the default Golgi position was completely neutral. The Golgi neither consistently leads or follows the nucleus and the speed and persistence of migration is the same regardless of position.

This default neutral state can be modified by altering the environment. Golgi positioning in single cells becomes more biased and more migratory after cells are treated with TGF-β, an inducer of EMT that is found at higher concentrations in the tumor microenvironment. This is evident in the correlation between enhanced migratory performance and the greater duration of the Behind Golgi state. The Golgi is now more frequently in the rear of the cell and cells migrate more persistently in this configuration. This shows that, in both single and group migration, consistent Golgi positioning is associated with more effective migration. The difference is, in group migration the consistency is inherent, and in single cell migration consistency must be created.

This need to add factors to build up to consistent positioning in single cell migration highlights the utility of studying individual cell migration. Factors that were
once inherent parts of a system can now be added, subtracted or modulated as necessary. Systems, like the one designed for this work, also illustrate the ease by which unintentional biases can influence the migratory system. Due to the minimalist nature of the system, these unknown influences are easier to ferret out so the cellular environment can be fully engineered and controlled.
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