TRANSLATING CELL MIGRATION: BASIC SCIENCE TO CLINICAL APPLICATIONS

by

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Abstract

Cell migration underlies many important physio- and pathological processes, including embryonic development, tissue homeostasis and wound healing, immune function, and cancer cell metastasis. Mechanistic understanding of cell migration in physiological contexts contributes to our ability to engineer tools for healthcare related to these phenomena. Metastasis, the process by which cancer cells spread from the primary tumor to distant parts of the body, is responsible for ~90% of cancer-related deaths. Advances in intravital imaging have illustrated that cancer cells migrate through confining tunnel-like tracks in the tumor microenvironment and are exposed to external physical forces. Confined cells can migrate using several distinct mechanisms, increasing the difficulty of preventing metastasis.

Following a discussion of our current understanding of confined cell migration and cancer progression, we apply bioengineering principles to study the intersection of cell migration and shear stress, as occurs during cellular intravasation into the circulation. We use microfabrication techniques to create a microfluidic model in which cells migrate through microchannels and then reach a larger orthogonal channel where fluid flow causes physiological rates of shear stress. Primary human dermal fibroblasts migrate in the microenvironment surrounding the vasculature but do not intravasate under normal physiological conditions. In line with this setting, we show in vitro that low shear stress levels cause migrating fibroblast to reverse their migration direction to avoid exposure to shear flow. Our data suggest a mechanism whereby shear stress increases intracellular calcium by opening mechanosensitive ion channels, in turn activating RHOA and myosin contractility, which together with CDC42 reverse the cells’ direction of migration. In contrast, cancer cells derived from fibroblasts (fibrosarcoma), exit the microchannels into shear flow. Compared to fibroblasts, fibrosarcoma cells have altered
expression of mechanosensitive ion channels, and reduced sensitivity to calcium signaling, which may contribute to the ability of these cells to enter the circulatory system during metastasis.

We next apply our knowledge of cancer cell migration to develop a diagnostic tool for prognosis and precision care of breast cancer, the Microfluidic Invasion Network Device (MIND). The MIND assay simulates aspects of the complexity and variety of cross-sectional areas of the tissue channels that cancer cells migrate through in vivo. Using this technology, we quantify the relative abundance of migratory and proliferative cells within heterogeneous cancer populations and demonstrate that these indices predict the metastatic potential of these populations with high accuracy. Highly-motile cells isolated from MIND exhibit increased metastatic potential in vivo compared to unsorted cells. RNA sequencing of highly-motile cells reveals an enrichment of motility- and survival-related genes. Lastly, we demonstrate the possible application of MIND for rapid screening of potential anti-metastatic therapeutics, which could allow for advances in the personalized medicine for breast cancer. Overall, our results highlight the critical role of the physical microenvironment in regulating cell migration and how this knowledge can be translated into tools for cancer treatment.

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Chapter 1

Introduction

Metastasis is responsible for the vast majority of cancer-related deaths, and is estimated to be responsible for 90% of the deaths of patients with solid tumors (1). The lethality of cancer’s spread throughout the body is caused by the increasing regions of the body where cancer cells disrupt normal bodily function, and the exponentially increasing challenge for physicians to locate and kill cancer cells with acceptable damage to normal tissue (2, 3). Metastasis is a complex process that is thought to involve the invasion of tumor cells into local tissue surrounding the tumor, intravasation (forced entry through the endothelial barrier) into blood or lymphatic vessels, transit of these cells throughout the body via the circulatory system, arrest of the cells on the walls of a circulatory vessel, extravasation (forced exit across the endothelial barrier), invasion into and colonization of a secondary site, and proliferation of a secondary tumor (3-5).

Migration of cancer cells, and numerous other cell types that interact with cancer cells during metastasis, plays an important role during this process (5). Cell migration was initially studied in two-dimensions (2D) on glass coverslips, but most physiological settings for cell migration are in three-dimensions (3D) (6). Advances in intravital imaging have allowed researchers to visualize cancer cells as they metastasize within model organisms (7). Surprisingly, cancer cells migrate in vivo along topographical features that seem to provide the routes “of least resistance”. These cues are created by anatomical structures, including in and along blood vessels,
along ECM fibers, and through longitudinal tracks created between the basement membrane and tissues, and in the perivascular tracks of the brain parenchyma \(^{8-14}\).

Subsequently, significant effort has been put into developing physiologically relevant models of tumor cell invasion in an effort to better understand metastasis. Importantly, migration mechanisms observed in 3D are not predicted by the results of 2D studies \(^{15-19}\). Emerging relevant models include 3D collagen gels or Matrigel® to simulate porous ECM, hydrogel technologies to mimic the physical stiffness of the body, microfabricated 1D protein lines to simulate migration along ECM fibers, and microfluidic devices \(^{20, 21}\). Microfluidic devices provide precise control over the environmental dimensions (down to \(\sim 1 \, \mu m\)) and geometry, which allow researchers to closely mimic physiological architecture. Irimia et al developed one of the first microfluidic devices that simulated the physical confinement that cells experience \textit{in vivo} \(^{22}\). Surprisingly, they found that cancer cells spontaneously migrated in confining microchannels without any biochemical cues, while 3T3 fibroblast cells frequently changed their migration direction. These results suggest that physical confinement alone is enough to stimulate persistent migration in one direction, and that cancer cells may be more receptive to this physical cue.

Since then we have increased our awareness that a multitude of physical cues influence many stages of the metastatic cascade \(^{23}\). Early in this process, tumor cells invade the local ECM through tissue microtracks and then intravasate into local blood or lymph vessels. \textit{In vivo} models of these processes are complex and make it difficult to isolate the underlying mechanisms behind specific phenomenon \(^{24}\). Meanwhile, most \textit{in vitro} platforms for studying intravasation oversimplify the process by failing to contextualize it following local migration/invasion through physiologically relevant 3D-like tracks \(^{25, 26}\). A major physical cue during intravasation is the shear stress – or physical pressure per unit area caused by a change in the velocity of the blood flow from the vessel wall to the circulation \(^{23}\). Cancer cells must overcome this force to enter the circulatory system. Herein, we designed a microfluidic model system to study the intersection
of the physical cues of topographically-directed cell migration through a longitudinal track followed by entry into an orthogonal channel with shear flow, in order to see which of these cues dominate during cell intravasation. The physical stimulus of shear stress causes divergent responses between fibroblast and fibrosarcoma cells (a cancer arising from connective tissue cells), potentially contributing to cancer cells’ ability to intravasate in the presence of shear flow while most fibroblast cells turn around and remain within the channels.

We next sought to apply our lessons learned from the study of physiologically relevant confinement migration in the lab to address a major problem in the clinic. Current breast cancer care suffers from over-treatment with harmful therapies, as well as a poor feedback about whether a patient is indeed responding to treatment (3, 27). Current technology for prediction or early detection of breast cancer metastasis is limited to gene expression profiling (28) and the identification of circulating tumor cells (CTCs) (29) or DNA shed by tumor cells (ctDNA) (27) in the patient’s bloodstream. Together, these technologies suffer from the inability to predict metastasis with sufficient lead time to prevent metastasis with treatment, and difficulty in predicting response to therapeutics. We addressed this problem by developing a Microfluidic Invasion Network Device (MIND) that mimics the topography and confinement experienced by metastasizing cancer cells in order to identify patient tumor populations with a low versus high risk of metastasis. The percentage of highly migratory and proliferative cells correlates well to the metastatic potential of established breast cancer cell lines with known in vivo metastatic potential, engineering breast cancer cell lines with demonstrated metastatic potential, and specimens from clinically-relevant patient-derived xenografts. These results demonstrate that our simple, phenotypic approach to identify metastatic tumor populations based on their ability to migrate in physiologically relevant models of confinement overcome many obstacles faced by previous diagnostic tools. MIND is a potentially valuable clinical tool for prognostication and determination of patient-specific therapies for precision medicine.
We conclude by discussing future directions for understanding the intersection of physical and biochemical cues during cancer metastasis, pathways to validation and commercialization of MIND for clinical use in breast and other solid cancers, and the potential to develop the next generation of microfluidic devices for high-throughput screening of pathways involved in cell migration. In sum, this dissertation reviews and illustrates the application of confined migration assays to answer basic science questions and to apply this knowledge to improve human health.
Chapter 2

Getting from point A to point B: how cells migrate

The movement of cells plays an important role in both the development and the maintenance of multicellular organisms. Cell migration is fundamental to many physiological and pathological processes, including embryonic development, wound healing, immune response, and cancer metastasis (30). Highly-coordinated movement of cells during embryonic development is required for the proper formation of distinct tissues (31). Once fully matured, tissues are maintained by migrating stromal cells such as fibroblasts, which produce and remodel the extracellular matrix that supports living tissue and are required for both tissue homeostasis and wound healing. Fibroblast cells are stimulated to move to remodel or repair tissue (32), and their molecular aging and senescence (a halt of proliferative capacity), although poorly understood, is known to contribute to declining tissue function and repair in aged organisms (33-37). Tissue wound healing requires the migration of immune cells that trigger inflammation response, fibroblast and myofibroblast cells that contract the extracellular matrix (ECM) to help close the wound, and epithelial/endothelial cells to recover the wounded surface (25, 30, 38). Immune response involves the migration and intravasation/extravasation of many cell types in complex microenvironments, such as the direct and rapid migration of leukocytes to sites of damage or infection (39). Altogether, understanding of the complex methods by which cells move can contribute to our ability to engineer solutions to a wide array of physiological problems.
2.1 Cell migration in response to topographical cues

Sophisticated intravital imaging has been used to study the environments in which tumor and normal cells migrate \textit{in vivo}. Accumulating evidence demonstrates that an important mode of migration is through tunnel-like longitudinal tracks. This geometry occurs in the interstitial spaces of the tumor stroma (9-11), blood or lymph vessels of the circulatory system local to the tumor (40) or in these vessels at distant organs (40, 41). Tumor cells are consistently found to migrate along ECM fibers, and along (the outside) or within circulatory vessels (40, 42-44). This raised the question of whether longitudinal spaces occur in healthy tissue or are created by tumor cell remodeling of the ECM and induction of angio- and lympho-genesis. Interestingly, intravital imaging shows these spaces occur in healthy tissues (11, 45). The ECM contains pores ranging from <1-20 µm in diameter, as well as larger-scale structures that are not captured by homogenous 3D \textit{in vitro} gels (45). Larger-scale features occurring in the body, but not in 3D gels, include longitudinal tracks between the basement membrane and connective tissue, in and along blood vessels, along ECM fibrils, between adipocytes, and in the perivascular spaces of the brain parenchyma (9-14). These \textit{in vivo} pores/tracks range in cross-sectional area from 10 µm$^2$ to 300 µm$^2$ (11, 45) and can be 100-600 µm in length (8, 11). This \textit{in vivo} architecture plays a role not only in cancer metastasis, but in tissue remodeling (32) and immune cell function (46).

2.2 Engineered models of cell migration

Traditionally, cell migration has been studied in 2D on glass slides or tissue culture dishes, due to the omnipresence of these materials in labs and the ease of imaging cells in this context (47). These materials are quite stiff, with an elastic modulus (ratio of stress to strain) on the order of gigapascals; in contrast tissues in the body tend to be much softer, ranging from tens or hundreds of pascals (blood, brain, and lung), to ~1000 pascals (breast and skin tissue), several to tens of kilopascals (connective and muscle tissue), to bone, which has a similar elastic modulus.
to plastic/glass (48). Thus, most of the environments in which cell migration is contextualized occur on relatively soft substrates. This has been modeled extensively by coating glass or plastic vessels with hydrogels, such as polyacrylamide, that have tunable stiffness (49-51). Unfortunately, much of what we have learned by studying cell migration in 2D does not translate to results in vivo or in the clinic (15-19).

Migration along tracks created by thick, aligned bundles of collagen or other fibrous ECM proteins guides tumor cells away from the primary tumor (10, 52). This activity is clinically relevant in breast cancer, where alignment of ECM fibers perpendicular to the tumor guides cancer cell dissemination (52), and is an independent predictor of poor prognosis (53). Microfabrication techniques exist to create “one-dimensional” (1D) lines of protein on flat substrates in order to mimic migration along these fibers (54). Unfortunately, when used to predict the aggressiveness of clinical cancer specimens, these 1D patterns fail to predict the survival of glioblastoma multiforme (GBM) patients, indicating that they are not a suitable model of the complex local invasion of the brain parenchyma and corpus callosum that lead to death from the progression of this disease (55). As mentioned above, 3D uniform gels have been used to study migration in higher dimensions, but fail to recapitulate the higher-order architecture of anatomical features (45) navigated by cancer and immune cells. To overcome this limitation, advanced laser-based micropatterning techniques have been developed to control the architecture within 3D hydrogels, but are still in their early stages and remain limited by low-throughput and the requirement of advanced microscopy tools (56-58).

Replica molding of longitudinal tracks in polydimethylsiloxane (PDMS) has proved to be a robust model of cell migration. PDMS is optically clear and allows diffusion of oxygen and other gases to support cell culture (59-61). Molds for PDMS in complex geometries with a high-degree of accuracy can be created with standard photolithography techniques (62, 63). Replica molding of these patterns in PDMS and then bonding to a glass or PDMS-coated slide creates a 3D-like environment with highly-ordered geometry that is compatible with standard microscopy
techniques (63). Many subsequent studies of cell migration using these models have demonstrated their relevance to physiological cell migration (8, 18, 21, 22). Importantly, in addition to precise control of the dimensions, PDMS models of cell migration can be coated with various ECM proteins at differing concentrations (16, 22) and can be designed to integrate other cues, including biochemical gradients (64), osmotic gradients (15), shear stress (65), electrical fields (66), and the presence of other cell types (67). Altogether, PDMS-based microfluidic systems are a versatile platform that enable the accurate modeling of physiologically relevant cell migration from numerous contexts.

2.3 One foot in front of the other? How cells generate force in order to move

Cells move by generating force against some component of their environment, which results in the translocation of the cell body. Interestingly, varying circumstances cause cells to employ diverse mechanisms of generating this force. This wide range of abilities is less surprising when considering the context of human migration. Under normal circumstances, humans move by walking: we place one foot in front of the other, and then as we transfer our weight from our back foot to our front foot, frictional force is generated against the ground, causing our body to move forward. While walking on two feet is the easiest mode of migration on flat ground with no barriers, humans may be forced to use other mechanisms to migrate in different environments. For example, in environments with a low ceiling, we may have to crawl on our hands and knees. To move vertically relative to our environment, we can “crawl” upwards by climbing using hand- and footholds. In the absence of sufficient holds, climbers can use a mechanism called “chimneying” where they insert themselves into vertical cracks and press against the sides in order to generate frictional force that allows them to work their way upward. Lastly, in water, humans can swim, where they drive the surrounding water backwards to generate forward
momentum. What these mechanisms of human migration have in common is that they use the same components of the body (e.g., feet, legs, arms) but coordinate these parts in different patterns in order to generate force against the environment.

Similarly, cells integrate cues from their environment in order to generate force in a variety of ways. On 2D environments, cells simulate walking by cyclical generation and then disruption of adhesions at their leading and trailing edge (front and back) (47): cell “feet” are formed when transmembrane receptors from a protein family called integrins attach to the extracellular matrix. In front of the lead adhesions, the cells form a lamellipodia, a broad region where actin filaments polymerize (assemble and extend), pushing the membrane forward. This growing network of actin filaments creates new regions of contact between the cell and its substrate, where new adhesions form. As the leading edge progresses, long assembled actin fibers called stress fibers associate with myosin motors and contract, pulling against the adhesions at the cell leading and trailing edges to generate tension. Finally, this tension, along with biochemical signaling from focal adhesion kinase (FAK) and proteolytic activity of calpains coordinate to disassemble the adhesions at the trailing edge of the cell, allowing the now unbalanced tensional force across the cell to drag the rear of the cell forward (47).

Physical confinement caused by migration in 3D environments does not support the formation of broad lamellipodia that is characteristic of migration in 2D. In 3D, cells can generate physical force by forming narrower lamellipodia at the leading edge, which are driven forward by actin polymerization (68). HT-1080 fibrosarcoma cells can migrate in soft 3D collagen gels without forming adhesions to their surrounding environment (69). When confined cells cannot adhere to their environment, they are able to generate friction against the surrounding structures to propel themselves forward, which has been termed “chimneying” after the example of the climber give earlier (70, 71). Cells in confinement can also use contractility-based blebbing or amoeboid modes of migration, where contractile cytoskeletal force increases intracellular pressure by circling around the cell (68) or by pulling the nucleus forward like a piston (72), and
this increased pressure relative to the environment pushes the cell forward. Cells can also move by the osmotic engine model, which is characterized by a net influx of water and ions at the cell leading edge and a efflux of water at the trailing edge that propels the cell forward (15, 73). Lastly, macrophages, and perhaps other cells, can “swim” via RhoA-driven rearward flow of the cell membrane, which generates frictional force against the relatively viscous surrounding liquid media to propel the cell forward (74).

2.4 Ion signaling and ion channels in cell migration

The cellular components involved in migration are regulated by complex and overlapping mechanisms. One of these mechanisms is by the control of ion concentrations within the cell by different protein transporters and channels that can allow charged ions to pass through the hydrophobic cellular membrane (75). Ca\(^{2+}\) plays a critical role in regulation cell migration, which is also affected by K\(^+\), Na\(^+\), Mg\(^{2+}\), and Cl\(^-\) (75, 76). Ion transporter activity and ion concentrations are co-dependent on each other, as many ion channels are sensitive to pH or Ca\(^{2+}\). The concentration of individual ions can also be co-dependent, such as in the case of Ca\(^{2+}\) and H\(^+\), which cannot change independently (77).

The actin cytoskeleton plays a critical role in most modes of cell migration. Assembled actin filaments can to myosin and to generate forces which are transmitted via focal adhesions to the ECM (78). Both cytoskeletal and adhesion dynamics are regulated by the concentration of ions within the cell and by the activity of ion transporters (76). Actin monomers reversibly assemble into filaments, and these dynamics are regulated by proteins that promote actin polymerization by nucleating new filaments or new branches off of existing filaments (such as the Arp2/3 complex), and by proteins such as cofilin that inhibit actin polymerization by binding to actin monomers or severing existing actin filaments (79). Cofilin-mediated actin severing produces free barbed ends that can allow for dynamic re-assembly or redistribution of actin. Thus,
cofilin plays a major role in actin dynamics and membrane protrusion at the cell leading edge and in the tip of invasive structures, which can promote actin-dependent cell migration and invasion (80). Cofilin binds F-actin during local decreases in the intracellular pH (81); in the lamellipodium this increase is mediated by the sodium hydrogen exchanger, NHE1, which exchanges H+ ions within the cell for extracellular Na+ ions, decreasing intracellular H+ concentration and thereby increasing the pH (82, 83). Another actin-binding protein involved in filament assembly and disassembly is gelsolin, which like cofilin is activated by local acidification (84). Altogether, local NHE1-mediated intracellular acidification plays a large role in regulating actin cytoskeletal dynamics. Unsurprisingly, NHE1 is required for efficient cancer cell migration (15, 85). In light of this role, the fact that NHE1 is upregulated by several tumor types (86, 87) suggests that this transporter plays a role in cancer progression and metastasis.

Calcium is an important second messenger in the cell, which maintains a ~10,000-fold gradient in extracellular to intracellular calcium concentration by sequestering this ion in organelles or pumping it out of the cell (88). Due to their low intracellular Ca2+ concentrations, many cell types are sensitive to calcium signals. Calcium concentration has a great impact on cytoskeletal dynamics because Ca2+ interacts with numerous proteins that play a role in cell contractility and adhesion. Myosin II (89), myosin light chain kinase (90), calcium/calmodulin-dependent protein kinase II (91), focal adhesion kinase (92), and calpain (93) are influenced by Ca2+. Ca2+ regulation is well studied in the context of its role in triggering muscle fiber contraction, which occurs when increased Ca2+ binds to calmodulin to activate myosin light chain kinase (MLCK), which in turn phosphorylates the regulatory myosin light chain on serine19 and causes the myosin motor to contract against bound actin filaments (94, 95). This mechanism also regulates blood vessel tension via smooth muscle cells (96). Ca2+-activation of myosin II generates the contractile force that aids in trailing edge retraction in migrating cells, which suggests increased Ca2+ activity at the rear of migration (97).
Along these lines, a trailing edge-leading edge Ca\(^{2+}\) gradient (higher at cell rear) has been observed in migration cells and is thought to coordinate the activities of these migration-related molecules (75, 98). Despite this global gradient, local transient Ca\(^{2+}\) signals have been observed and promote focal adhesion disassembly and regulate the direction of migration (90, 99, 100). Protein channels that mediate Ca\(^{2+}\)-entry into the cell such as TRPC6, play a role in the chemotaxis (migration up a gradient of biochemical signals) of murine neutrophils (101). TRPM7 mediates local Ca\(^{2+}\) flickers at the front of fibroblast cells chemotaxing towards platelet-derived growth factor (PDGF) and its inhibition prevents this directed migration (102). Ca\(^{2+}\) can also regulate transport of other ions, such as through the K\(_{Ca}\)3.1 channel. Through this link, activity of the TRPC1 and the K\(_{Ca}\)3.1 channel is required for cancer cell chemotaxis (103-106).

Increasing tension on the cell membrane activates mechanosensitive (stretch-activated) ion channels (107). Opening of these channels, such as the ones that belong to the Piezo and Transient Receptor Potential (TRP) families, leads to rapid influx of Ca\(^{2+}\) into the cell in response to the steep gradient of this ion (108, 109). Shape changes in the cell due to the generation of protrusions and squeezing into confined spaces trigger membrane tension (17). These shape changes also require the cell to regulate its volume, which is critical in cell migration and is mediated by the osmolarity of the cell and the extracellular media (76). Cells decrease their volume (a process known as regulatory volume decrease, RVD) by using K\(^+\) and Cl\(^-\) channels to transport these ions out of the cell, decreasing their osmolarity and triggering water efflux from the cell. Water transport across the membrane is expedited by protein water channels called aquaporins (110). Cell volume increases are accomplished by Na\(^+\), K\(^+\), 2Cl\(^-\) cotransport, Na\(^+\)/H\(^+\) exchange, and non-selective cation channels (76). As volume regulation is critical to cell migration, many volume-regulating channels play also regulate cell motility including TRP channels (99, 111), K\(_{Ca}\)3.1 (112), K\(_v\)1.3 (113), NHE1 (15, 114), NKCC1 (115) and aquaporins (110).
Chapter 3

Genetic and environmental factors driving cancer onset and breast cancer metastasis

Cancer is caused when the constraints that control the behavior of normal cells are deregulated. A review of cancer behaviors proposes that malignant growth requires the acquisition of 6 traits: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis (programmed cell death), unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis (116). Multicellular organisms control the proliferation and movement of cells through contact inhibition, which is an umbrella term encompassing two parallel processes: contact inhibition of proliferation (CIP) and contact inhibition of locomotion (CIL) (117). Disruption of these processes leads to the uncontrolled proliferation and migration of cancer cells.

3.1 Genetic and epigenetic perturbations driving cancer onset

During cancer onset, mutations and functional changes to the genome of precancerous cells drive cancer progression. Gene mutations are permanent changes to the DNA of cancer cells, which can in some cases silence or over-activate genes. Mutations in individual genes can be inherited at birth, occur randomly, or be caused by damage to DNA caused by environmental factors such as smoking. Cells can also permanently gain or lose entire chromosomes through rare mistakes during cell division, leading to daughter cells that have significantly more or fewer...
copies of many genes (118). Increasing evidence demonstrates the role of non-permanent changes to the genome of cancer cells, which are known as epigenetic changes. The most common epigenetic changes in cancer are DNA methylation, histone modifications (phosphorylation, methylation, and acetylation), and RNA-interference through noncoding strands (119). DNA methylation is mediated by DNA methyltransferases and typically leads to the repression of the methylated genes (120). Histones are globular proteins that act as spools for the organization of DNA, and their modification adjusts the physical accessibility to genes by winding (inaccessible) or unwinding (accessible) the DNA strand on which they are encoded. RNA-interference by micro-RNA (miRNA) leads to the post-translation disruption of gene expression (reviewed in (121)), while long non-coding RNA (lncRNA) regulate gene expression through multiple mechanisms (see (122)). Importantly, while epigenetic gene regulations do not cause permanent changes to DNA, they are heritably both mitotically and meiotically, meaning that these alterations can be passed to daughter cells, as well as offspring of multicellular organisms (123). Through a combination of these mechanisms, cells become cancerous when they have altered the expression of enough genes to acquire the above six abilities.

Genes that regulate cell growth under normal conditions are termed proto-oncogenes, while mutated forms of these genes that contribute to cancer onset are called oncogenes. The first oncogenes identifies were members of the Rat sarcoma, or Ras protein family, namely N-Ras, H-Ras and K-Ras (124). Ras proteins play a causal role in some of the most deadly cancers, including pancreatic, colon, and lung cancer where they are mutated at a rate of 91%, 42% and 33%, respectively (125). While activating mutations in the canonical Ras/MAPK pathway occur at a lower rate (2-10%) in breast cancer (126), this pathway’s activity is linked to breast cancer metastasis, and its aberrant activity may be induced by overexpression of upstream receptor tyrosine kinases such as EGFR and Her2, which is common in breast cancer (127). A common breast cancer proto-oncogene is phosphatidyl inositol 3 kinase (PI3K), which contributes to cell
survival, proliferation, and migration (fully half of the above cancerous traits) (128). In fact, the PI3K pathway is activated in greater than 70% of patients with invasive breast cancer (129).

Acquisition of a cancerous phenotype requires the altered gene expression of multiple genes (116). The lifetime likelihood of developing cancer is the product of the probabilities of acquiring each individual perturbation. As a crude example, if cancer onset required acquisition of 4 mutations, and the lifetime risk of each individual mutation was 10%, then the lifetime risk of cancer onset is $\frac{1}{10} \times \frac{1}{10} \times \frac{1}{10} \times \frac{1}{10} = \frac{1}{10,000}$. Inheriting genetic predispositions to cancer onset, such as breast cancer type 1 and 2 susceptibility proteins, increased the lifetime risk of developing cancer by a factor of the likelihood of acquiring that mutation; for the example above, this would increase the lifelong odds from 1/10,000 to 1/1,000. Unfortunately, actual quantification of these likelihoods is not straightforward as similar patterns of gene expression can be achieved by different combinations of genetic or epigenetic changes. Further, even within the same cancer, there are many patterns of gene expression, and this is an area of active study.

3.2 Colonization of tissue by cancer cells

Tumor cell growth and proliferation robs local cell of nutrients such as glucose and oxygen, which can reduce their functionality (130). In fact, by the time tumors reach 1-2 mm$^3$ in size, they are limited by nutrient and oxygen availability, and their progression relies on induction of the formation of new blood vessels in a process known as angiogenesis (131). Tumor cells induce angiogenesis by secreting vascular endothelial growth factor (VEGF) (130). VEGF also increases the permeability of blood vessels as they remodel, which contributes to cancer cell intravasation during metastasis (131). Further, angiogenesis during tumor progression increases the proximity between tumors and blood vessels (132, 133). The presence of larger diameter microvessels, where shear stress is reduced due to lower flow velocity, in human colorectal tumor tissue has been linked to greater incidence of liver metastases in patients (134).
Growth of breast cancer tumors are often driven by hormone and/or growth factor signaling. The estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (Her2) are frequently overexpressed by breast tumors (118). Upregulation of these receptors leads to hypersensitivity to physiological levels of their ligands and uncontrolled tumor cell proliferation. Her2 (also known as ErbB2) is upregulated in ~20% of breast cancer patients, leading to increased proliferation, escape from apoptosis, and migration (135). This has led to the development of successful Her2-targeting therapies, such as Herceptin® (trastuzumab). In fact, we have shown using the methods described in this dissertation that even single allele copy activating mutations of Her2 cooperate with commonly observed PI3K mutations to enhance the motility of breast epithelial (MCF-10A) and breast cancer (MCF7) cell lines in 2D wound healing assays and in microchannels (136).

3.3 Interstitial fluid flow in the tumor microenvironment

Interstitial flow is the movement of fluid through the extracellular matrix that occurs in all living tissues (130, 137, 138). This flow begins as blood plasma leaks from capillaries, flows through the interstitial space, drains into lymphatic vessels, passes through the lymph nodes, and finally returns to veins (130, 137). This convective transport of fluid is driven by Starling forces, or the net differences in hydrostatic and osmotic pressures between the intravascular and the interstitial spaces (130). Simplistically, in the arterial capillaries, the hydrostatic pressure difference between the blood vessel and the interstitial space outweighs the osmotic pressure gradient and thus drives fluid flow into the interstitium; conversely, at the venous end of the capillary bed, osmotic force due to blood plasma proteins (many of which are too large to pass out of circulation) drives fluid back into circulation against the hydrostatic gradient (the magnitude of which is reduced here due to the lower venous pressure) (139). Direct measurement of the interstitial fluid flow velocity is challenging, but has been performed in animal models
using fluorescence recovery after photobleaching (140), circulation of quantum dots (141), and in both humans and animal models using magnetic resonance imaging (MRI) (142, 143), and agree with mathematical modeling (144). These separate studies generally concur that the normal physiological velocity is 0.1-10 µm/s, but that range is altered significantly by wound and disease states (130).

Interstitial flow provides mass transfer between the circulation and tissue. Such transfer provides oxygen and other nutrients to the tissue, removes waste products, and functions in immune response (130, 137). Interstitial flows provide nutrient transport and a means of cell-cell communication over relatively long distances when diffusion is inadequate (145). These distances result in gradients of growth factors from the VEGF, FGF, Wnt and TGF families, as well as immunosuppressive chemokines such as CCL19 and CCL21, which influence cell behavior and can bind to ECM components to create long-lasting signaling effects (146, 147). Cell migration can follow these gradients, a process known as autologous chemotaxis, which influences physiological and pathological process including immune cell homing (130, 148) and tumor (glioma, breast, melanoma) (149, 150) cell and cancer-associated fibroblast (130) trafficking to lymph nodes.

Tissue maintenance, remodeling, and wound healing are all affected by interstitial flow. Force from this fluid transport (in addition to solid stress) contributes to matrix protein production in cartilage (151) and bone (152). Flow leads also to stiffening of existing or newly deposited ECM through several mechanisms. Shear stress from fluid flow acts on cells to promote contractility through multiple mechanisms (68, 153-155), which leads stromal cells to generate tugging forces that align matrix fibers (52, 155, 156). Exposure to shear stress can also lead fibroblast cells to differentiate into contractile myofibroblasts (157, 158) that exert force on the ECM. Fluid flow itself has been show in vitro and in vivo to trigger the release of matrix metalloproteinases (MMPs) that aid cells in degrading and remodeling the ECM (159-162). The combination of contractile forces and matrix degrading proteases allows for substantial ECM remodeling and bundling. Further, the ECM functions as a repository for several growth factors.
(163-165), including transforming growth factor-β (TGF-β), which under force can be released to alter their signaling potency (158), a mechanism that can be activated by both fluid shear and shear-induced contractile forces. Amongst its many roles, TGF-β causes fibroblast and myofibroblast cells to contract, which generates a tugging force on ECM fibers that can pull them into alignment together, causing the matrix to become stiffer (166, 167). In addition to improving the accessibility of TGF-β already bound to the ECM, several in vitro studies have shown that relatively high velocity flows of 4-10 µm/s through collagen matrices containing human lung or dermal fibroblasts increases their production of TGF-β1, leading to increased collagen production, differentiation into myofibroblasts, and alignment of collagen fibers (157, 168, 169).

Tumors generate increased hydrostatic pressure that drives higher interstitial flow rates (170, 171). Magnetic resonance imaging (MRI) performed in mice demonstrates the rates of interstitial flow ranged from 0.1-0.5 µm/s and correlated to the diameter of the tumor (142). High tumor interstitial fluid pressure yields to fast drainage rates into tumor-associated lymphatic vessels during in vitro characterization, suggesting that lymphatic intravasation is aided by high interstitial pressure (172). Indeed, up to 20x the rate of lymphatic drainage was observed by visualization of quantum dots in mice bearing tumors compared to healthy mice (141). In fact, metastasis of breast cancer cells to lymph nodes is correlated to rates of interstitial fluid flow (173). Combined with tumor driven angio- and lympho-genesis, as well as tissue remodeling induced by shear stress, interstitial flow promotes tumor cell dissemination from the primary tumor to circulatory vessels (130).

### 3.4 Intravasation, survival in the circulatory system, and extravasation

Cellular intravasation plays an important role in development, immune cell function and cancer metastasis (25). For a cell to enter a circulatory vessel, it must cross the tightly-formed layer of endothelial cells (96) and resist the exposure to shear force from the blood flow (25).
Vascular endothelial cells maintain blood vessel integrity by forming tight junctions via the transmembrane protein zonula occulens-1 (ZO-1) and adherens junctions with vascular-endothelial cadherins (VE-cadherin) (174). Disruption of endothelial cell-cell junctions may be a key mediator of intravasation (25). In fact, human tumor cells were found to intravasate in a zebrafish model only in locations where the vasculature was being remodeled, and not at intact vessels (175). Tumor-associated blood vessels are leaky due to disruption of normal vascular function by paracrine signaling (such as secretion of tumor necrosis factor alpha, TNFα) (176, 177), protease degradation (24, 178-180), induction of remodeling/angiogenesis (130, 181), and hypoxia (182, 183). In mouse models of mammary carcinoma, tumor-associated leaky vasculature frequently had intercellular holes between endothelial cells averaging 2 µm in diameter and occurring up to 5 µm in diameter. Tumor cells also exert force to cross the endothelial layer, either by rearranging their cytoskeleton to compress internal cell components (23) or by cell division to produce outward force to break endothelial junctions (184). Along these lines, in vitro studies reveal that breast cancer cells crossing the endothelium create disruptions of ≥ 20 µm in width (184).

Shear rates during parts of circulation, such as in the heart, can exceed 1000 dyne/cm², which can induce cell apoptosis (185, 186). Metastatic cancer cells must resist apoptosis to eventually exit the circulatory system. In fact, circulating tumor cells (CTCs) from breast (MDA-MB-231) or prostate (PC-3) cancers can resist shear-induced apoptosis compared to normal cells via Rho-kinase-mediated actin rearrangement (185).

Following survival in the circulatory system, CTCs must halt their flow by arresting on blood vessel walls. Tumor cells initiate contact with the endothelial cells of the blood vessel wall by expressing binding partners (ligands) of a class of cell surface receptors called selectins (187). CD44 is a cell adhesion molecule that plays a distinct role in colon and breast cancer metastasis and serves as a function P-selectin ligand (188-190). Relatively weak bonding and transient bonding of selectins initiates tumor cell rolling along the endothelial cell layer (as these weak
bonds form break and reform), which slows the cell velocity and orients them close to the surface (191). Subsequently, firm adhesion to the endothelial layer and extravasation is mediated by integrin receptors (192). This occurs most frequently in post-capillary venules, which experience lower shear rates and increased expression of selectin receptors that tumor cells can bind to.

3.5 Breast cancer metastasis

Cancer metastasis is responsible for the vast majority of breast cancer-related deaths (1, 4). Localized breast cancer has a 99% five-year relative survival rate, which drops to 85% in patients where the disease has spread regionally, and to 27% in patients with distant spreading (1, 193, 194). Thus, it is critical to assess which patients are at risk of developing metastasis in order to provide effective treatment. Current estimates are that 20-30% of breast cancer patients with early stage disease will eventually develop metastatic recurrence (195); this estimate is corroborated by the fact that the 15-year relative survival rate for breast cancer is 80%, with the potential for an increased percentage of deaths at time points beyond fifteen years (193). In the year 2018, approximately 266,000 women will be diagnosed with breast cancer in the United States (196). During this same time frame, it is estimated that over 50,000 women will develop de novo metastatic breast cancer or develop metastatic recurrence from a previously diagnosed breast cancer (197). Incidence rates for male breast cancer are roughly 10% the values of those in women (194). Breast cancer mortality has declined from 32 in 100,000 to 21 in 100,000 the last 35 years, largely due to increased mammography screening (which has coincided with identification of a larger proportion of early-stage breast cancers) (198). Current breast cancer care suffers from over-treatment with harmful therapies, as well as a poor feedback about whether a patient is indeed responding to treatment (3, 27).
3.5.1 Breast cancer molecular subtypes, prognosis and treatment options. Subtypes of breast cancer have long been identified by their histological features. Specifically, subtypes of breast cancer exist that are characterized by overexpression of progesterone receptor (PR), estrogen receptor (ER), or Her2, deemed PR+, ER+, or Her2+. Lack of overexpression of all three identifiers was noted to correlate to poorer prognosis, and deemed triple negative breast cancer (199). Gene expression studies of breast cancer specimens from across these subtypes confirm the existence of distinct breast cancer subtypes, but reveal more complexity than previously thought (200). Specifically, five breast cancer molecular subtypes are now broadly recognized based on their grouped clustering based on gene expression profiles: luminal A, luminal B, Her2+, basal, and normal-like (200-202). These molecular subtypes have distinct clinical identifiers and outcomes.

Luminal A breast cancers make up about 20% of breast cancers, tend to have a low grade (not significantly differentiation in morphology from healthy tissue) and are ER and/or PR positive, Her2-, and Ki67- (<14% of cells bear this marker for proliferating cells) (203, 204) and tend to have good outcomes. About 40% of breast cancer is Luminal B, which is subdivided by whether or not the tumors are Her2+; ER and/or PR positive, Ki67+, and Her2- have intermediate prognosis and tend to be grade 2-3, while luminal B cancers that ER and/or PR positive, Her2+ and Ki67± have poor prognosis. Her2 over-expressing breast cancers are ER-/PR-/Her2+, consist of about 10% of breast cancer and have higher grades (2/3) and poor outcomes. Basal breast cancer represents 10% of patients and has the triple-negative histological staining and correlates to high grades (3) and worse outcomes. Finally, normal-like breast cancers are named for sharing some gene expression signatures with normal breast tissue, but otherwise remain poorly defined; normal-like breast cancers are ER+/PR+/Her2-/Ki67-, can be of any grade and have intermediate outcomes, largely due to heterogeneity within this subtype. As normal-like breast cancers share the same histological features as luminal A cancers, they were not historically recognized and received the same treatment. However, advances in gene sequencing have revealed that these two
subtypes have significantly different gene expression patterns, which perhaps explain the poorer outcomes for normal-like as compared to luminal A cancers (205).

The unique characteristics of certain breast cancer molecular subtypes offer the opportunity for targeted therapies against the cancer cells. PR and ER positive breast cancers rely on overactivation of these receptors to increase their sensitivity to body hormones, increasing the downstream proliferative signaling from these receptors. Thus, these subtypes are typically treated with hormonal therapy that functions to reduce the sensitivity of these cancers to the hormone, estrogen in order to reduce its potent proliferative effects on tumor cells (206). Hormone therapies adopt one of two strategies to reduce estrogen signaling: competitive binding partners that bind to the estrogen receptor but do not induce downstream signaling, or estrogen-deprivation therapy that employs aromatase inhibitors to reduce the body’s natural production of estrogen (206). Estrogen-targeting hormone therapy is also an effective strategy in PR+ patients as the progesterone receptor is a major downstream target of ER (207-209).

Although Her2+ cancers tend to have poor prognosis, drugs that specifically target tumor cells from this subtype have been developed. The first anti-Her2 therapy, trastuzumab (marketed as Herceptin®), was first approved by the FDA in 1998. Trastuzumab is a mouse monoclonal antibody that binds to the extracellular domain (ECD) of Her2. The mechanism by which this leads to cancer cell death is not completely understood, but is likely due to the combined effects of immune cell targeting of the antibody-bound cancer cells (antibody-dependent cell-mediated cytotoxicity) (210), decreased DNA repair, intracellular signaling, anti-angiogenic effects (211). Another FDA-approved monoclonal antibody for targeting Her2, pertuzumab (Omnitarg®), binds to a different site than trastuzumab and has shown to have additive effects in combination (212). Other anti-Her2 therapies, such as lapatinib (Tykerb®) block target downstream Her2 signaling by inhibiting its tyrosine kinase activity (212). While these drugs have somewhat improved outcomes in this aggressive subtype, tumor drug resistance and metastasis continue to limit clinical success.
Basal-like breast cancers are triple-negative, and thus lack overexpressed surface growth factor receptors that can be targeted in therapy. That leaves only general therapies, such as chemotherapy and radiotherapy. In addition to the lack of targeted therapeutic options, this subtype tends to be more aggressive and metastatic, leading to worse clinical outcomes (205).

Drug resistance, both to chemotherapy and targeted therapies, is a major problem in breast and other cancers. ER-positive breast cancers treated with hormonal therapies respond by activating ER signaling through numerous mechanisms, including phosphorylation-dependent and androgen metabolite-dependent mechanisms, which demonstrates that even within subtypes of breast cancer, significant heterogeneity exists (206). Therapies are in development to target signaling pathways and biological processes that contribute to cancer metastasis such as against cell migration, proliferation, survival, and metabolism. Since the PI3K and Ras/MAPK pathways play a role in breast cancer progression and metastasis, they have both been targeted by numerous therapeutics; however, cross talk between these pathways has limited the success of targeting either pathway (128). The complex genotypes of tumors, even within the same subtype, makes response and resistance to therapy very hard to predict (27). These results highlight the advantage of a phenotypic assay to evaluate the efficacy of potential therapeutics in the complex and varied genetic landscapes present in tumor populations (213). \textit{Ex vivo} growth of patient-derived tumor cells in tissue culture dishes or as organoids has been demonstrated in principle as a platform for screening the effectiveness of anti-tumor drugs for patient-specific efficacy (213, 214). Unfortunately, many therapies are only effective in the short-term, following by resistance of the tumor, or disseminated metastatic cells to the drug (215, 216). Due to this acquired resistance, another option is to predict and inhibit the metastatic dissemination of tumor cells.

3.5.2 \textbf{Current challenges in predicting tumor metastatic potential.} Tumor metastasis compounds the issues associated with primary tumors by the eventual creation of secondary tumors at distant sites. This increases the number of local areas that must be treated in the clinic
and leads to the spread of cancer cells beyond the area that would be removed during a resection surgery for the primary tumor. Metastatic cancer cells acquire a repertoire of distinct abilities that enable them to separate from the primary tumor, locally invade the surrounding stroma, intravasate and survive in the circulatory system, roll and arrest on a vessel wall, extravasate, locally invade and form a pro-metastatic niche, and finally proliferate to colonize a distant organ (1, 8). Cancer metastasis is a highly inefficient process, and it is estimated that only 0.001% - 0.02% of cancer cells that enter circulation can form a metastatic tumor (217). Identification of these highly-metastatic cells is critical to development of methods for the prognosis and treatment of cancer metastasis.

Tumor populations are heterogeneous (2, 218, 219), and it is widely believed that only a small portion of cancer cells are capable of forming metastases (220-223). The identification and isolation of the subpopulation of cells responsible for cancer metastasis would aid in its prediction and treatment. Cancer stem cells (CSCs) are widely believed to be responsible for metastatic recurrence and resistance to chemotherapy (221, 224-226). Both CD44+/CD24−/low and ALDH1+ expression are considered to be markers for CSCs, and can be used to isolate cells with enhanced tumorigenic or metastatic potential (190, 225-229). Unfortunately, both of these markers have been met with mixed results when assessed in clinical samples. The first assessment of CD44+/CD24−/low populations in paraffin-embedded tissues of 136 patients revealed no association with clinical outcome, although it did predict metastasis in a small sample set (230). Zhong et al found that CD44+/CD24−/low and ALDH+ are both risk factors for recurrence and metastasis, but with low sensitivity (63.3% and 63.1%, respectively) (231). Two reports found that low CD24 expression indicates good prognosis in ER-positive breast cancer, indicating that the effectiveness of this biomarker may be subtype-specific, and require validation prior to use in each breast cancer subtype; these reports found that ALDH1 was either not predictive or rarely detected, rendering it less helpful diagnostically (232, 233). Due to the limitations of biomarkers for
metastatic cells in the clinical setting, in this dissertation we develop methods to use a functional assay for cell motility to identify cells with high metastatic potential.

Current predictors of breast cancer metastasis require further improvement. Gene expression profiles such as Oncotype DX measure the expression levels of a subset of genes and use the expression pattern to predict prognosis, and in some cases, likelihood of responding to treatment. However, it is unlikely that one panel will be effective for all patients because breast cancer progression can be caused by mutations in different pathways, at different levels within the same pathway, or even at different loci in the same gene (27). Due to the high cost of these tests (> $3,000), questions remain about the cost-effectiveness of their use in the clinic (234, 235).

Detection of CTCs using the FDA-approved CellSearch system has prognostic value for predicting disease free survival and overall survival (236-238). However, current implementation of the technology still suffers from low sensitivity and specificity for predicting patient outcomes (239-241). Detection of ctDNA is typically performed by sequencing of primary tumor DNA and then developing polymerase chain reaction (PCR) probes for unique characteristics of the tumor genome (e.g., somatic mutations or chromosomal rearrangement). This approach has been applied in early studies to predict the recurrence or metastasis of breast cancer with high specificity (up to 100%) but has been limited by its sensitivity to detect ctDNA (31-86%) (242-244). Of note, Olsson et al achieved 86% sensitivity by testing serial blood plasma samples for each patient but observed inconsistency from sample-to-sample in whether a patient with metastatic disease tested positive for ctDNA. Prediction of metastasis in this study was made with a median of about six months lead time in advance of clinically detection of metastasis. While minimally invasive, detection of both CTCs and ctDNA require serial sampling of the patient in order to be effective. Both of these methods have potential to monitor the response of a patient to treatment, but do not at this time offer a prediction of whether a patient will respond to specific therapeutic regimens (3, 27). Improved sensitivity, lead time in prediction of metastasis before its
clinical detection, and ability to screen therapeutic regimens for patient-specific effectiveness will improve patient outcomes.
Chapter 4

Fibroblasts don’t go with the flow: the intersection of confined cell migration and shear stress in normal and cancerous cells

4.1. Introduction

Cell invasion and intravasation are critical steps during immune response and cancer cell migration but have many unanswered questions due to the contribution of multiple biochemical and physical cues to each of these steps (6, 20, 245). While in vivo platforms are widely-used to study these processes, the complexity of these methods makes it difficult to isolate the underlying mechanisms behind specific phenomenon (24). Meanwhile, most in vitro platforms for studying intravasation fail to contextualize it following local migration/invasion through physiologically relevant 3D-like tracks (25, 26).

Cells migrate through the in vivo extracellular matrix (ECM) by both protease-dependent and -independent methods. Proteolytic degradation of the ECM by either secreted or membrane-bound matrix metalloproteinases (MMPs) creates tracks that cancer, stromal, and immune cells can traverse (246-251). In particular, degradation-dependent migration is critical when ECM pores fall below ~10% the size of the nuclear cross section (neutrophils, 2 µm³; T cells 4 µm³; tumor cells, 7 µm³) due to the cells’ apparent inability to deform its largest and stiffest organelle beyond this limit (252). Interestingly, degraded matrix tracks are utilized by opportunistic cells to navigate the ECM (253-255), demonstrating that protease-dependent migration can stimulate subsequent protease-independent migration. Protease-independent migration also occurs through
pores in the ECM, and through longitudinal tracks created by numerous anatomical features, such as between the basement membrane and connective tissue, in and along blood vessels, along ECM fibrils, and between adipocytes (9-14). Intravital microscopy characterization has shown that tissue microtracks have lengths on the order of 100-600 µm (8, 11) and widths from 3-30 µm (11, 45). Little experimental work focuses on the transition from migration in tissue tracks to entry into the vasculature system.

Cellular intravasation plays an important role in development, immune cell function and cancer metastasis (25). For a cell to enter a circulatory vessel, it must cross the tightly-formed layer of endothelial cells (96) and resist the exposure to shear force from the blood flow (25). Vascular endothelial cells maintain blood vessel integrity by forming tight junctions via the transmembrane protein zonula occulens-1 (ZO-1) and adherens junctions with vascular-endothelial cadherins (VE-cadherin) (174). Disruption of endothelial cell-cell junctions may be a key mediator of intravasation (25). In fact, human tumor cells were found to intravasate in a zebrafish model only in locations where the vasculature was being remodeled, and not at intact vessels (175). Tumor-associated blood vessels are leaky due to disruption of normal vascular function by paracrine signaling (such as secretion of TNFα) (176, 177), protease degradation (24, 178-180), induction of remodeling/angiogenesis (130, 181), and hypoxia (182, 183). In mouse models of mammary carcinoma, tumor-associated leaky vasculature frequently had intercellular holes between endothelial cells averaging 2 µm in diameter and occurring up to 5 µm in diameter. Tumor cells also exert force to cross the endothelial layer, either by rearranging their cytoskeleton to compress internal cell components (23) or by cell division to produce outward force to break endothelial junctions (184). Along these lines, in vitro studies reveal that breast cancer cells crossing the endothelium create disruptions of ≥ 20 µm in width (184).

Shear stress is the force per unit area caused by the movement of adjacent layers of fluid with different velocities. The shear stress in the circulatory system varies based on the pattern of blood flow, as well as the type and size of vessel in question. The average shear stress in the
venous circulation is 1-4 dyne/cm², compared to 4-30 dyne/cm² in the arterial circulation (23, 256). Smaller venules and arterioles can experience higher shear rates of ~20 or ~50 dyne/cm², respectively (257-259). Capillary shear stress has been estimated with computer modeling to be in the range of 1-95 dyne/cm² with an average of 5-20 dyne/cm² (260), which is in agreement with recent measurements in human conjunctival capillaries where the average wall shear stress was 15 dyne/cm² (261). Finally, from flow velocity measurements, the calculated shear stress in capillary sprouts, which are relevant in the context of angiogenesis, are relatively low, at 0.2-6 dyne/cm² (262). Shear stress presents a physical barrier to intravasation (23, 25) that may also influence which vessels can be infiltrated by cells. Tumor cell intravasation is logically more likely to occur in the near vicinity of the tumor. Further, angiogenesis during tumor progression increases the proximity between tumors and blood vessels (132, 133). The presence of larger diameter microvessels, where shear stress is reduced due to lower flow velocity, in human colorectal tumor tissue was linked to greater incidence of liver metastases in patients (134).

We designed a microfluidic model system to study the intersection of the physical cues of topographically-directed cell migration through a longitudinal track followed by entry into an orthogonal channel with shear flow. Directed cell migration was observed in collagen-I-coated microchannels of width, \( W = 10-50 \mu m \), and height, \( H = 10 \mu m \). These dimensions are consistent with those of tissue tracks observed in vivo, as well as with the dimensions of gaps created by tumor cells crossing the endothelial layer, simulating the cell surface area exposed to shear flow during this transition. Parallel to the microchannels are larger 2D-like channels (\( W = 400 \mu m \) and \( H = 50 \mu m \)) where fluid flow is controlled. Hydrostatic pressure-driven fluid flow was used to generate a shear stress of 0.5 dyne/cm² at the microchannel exits. To understand how these physical cues influence cell behavior, we compared the migration of dermal fibroblasts, cells that exist in the vascular microenvironment but typically do not intravasate under normal physiological conditions (32, 263, 264), to that of cancer cells derived from this type of cells (HT-1080 fibrosarcoma). We found that primary human dermal fibroblast cells were sensitive to
this shear stress and tended to reverse their migration direction to remain inside the microchannels (while consistently exited under static conditions). Surprisingly, fibrosarcoma cells were not shear-sensitive and exited the microchannels during both static and shear conditions. Fibroblast response to shear stress requires entry of extracellular calcium through the mechanosensitive ion channel, TRPM7. Increasing intracellular calcium is sufficient to trigger most fibroblast, but only a small proportion of fibrosarcoma cells to reverse their migration direction in the absence of shear stress. We demonstrate in fibroblasts that shear stress activates RhoA and increases the phosphorylation of myosin light chain, and that Rho-kinase and Cdc42 activity are required for shear stress response. Together, our data suggest a mechanism where shear stress increases intracellular calcium by opening mechanosensitive ion channels, in turn activating RhoA and myosin contractility, which together with Cdc42 reverse the cells’ direction of migration. Fibrosarcoma cells are able to overcome this shear sensitivity, which may play a role in their metastatic behavior.

4.2 Materials and Methods

4.2.1 Cells and cell culture. Human dermal fibroblasts (GM05565) were a gift from Dr. Denis Wirtz (Johns Hopkins University, Baltimore, MD). Newborn human foreskin fibroblasts (NuFF) were a gift from Dr. Sharon Gerecht (Johns Hopkins University, Baltimore, MD). Chinese hamster ovary cells (CHO) were purchased from ATCC. HT-1080 cells were a gift from Dr. Sean Sun (Johns Hopkins University, Baltimore, MD). Cells were maintained in tissue culture flasks at 37°C and 5% CO2. GM05565, CHO, and HT-1080 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies/Gibco) supplemented with 10% v/v Fetal Bovine Serum (FBS) and 1% v/v Penicillin/Streptomycin (Life Technologies/Gibco). NuFFs were maintained in DMEM with high glucose and without sodium pyruvate (11965092, Life
Technologies/Gibco) supplemented with 10% v/v FBS. Cells were passaged using 0.05% trypsin-EDTA (Gibco).

**4.2.2 Fabrication of microfluidic device.** The microfluidic device consisted of an array of parallel 10 µm x 10 µm microchannels that were 200 µm long. Perpendicular to the microchannels were seeding and media channels with a width of 400 µm and a height of 30 µm. The devices were created using standard multilayer photolithography and replica molding. Channel schematics were drawn in AutoCAD (Autodesk) and then transferred to darkfield photomasks created by patterning of chrome on glass (Photoplot Store). The 10 µm tall feature (microchannels) were created by spin coating (Laurell Technologies) a 10 µm tall layer of SU-8 3010 negative photoresist (Microchem) on a cleaned silicon wafer (University Wafer). Spun photoresist was soft-baked then exposed to ultraviolet (UV) light (170 mJ/cm²) via a mask aligner (EVG). The exposed wafer was hard baked, then uncrosslinked photoresist was removed with SU-8 developing. A second layer was then patterned by spin coating the same wafer with SU-8 3025 to a height of 30 µm. The wafer was soft baked then exposed to UV-light (250 mJ/cm²) through a photomask exposing the patterns of the seeding and media channels. After hard baking and development, wafers were passivated by treatment with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Pfaltz & Bauer) overnight in a vacuum desiccator.

Microfluidic devices were cast in polydimethylsiloxane (PDMS) by covering the molds with a 10:1 w/w mixture of elastomer and crosslinker (Sylgard® 184 Silicone Elastomer Kit, Dow Corning). Air bubbles were removed by incubation at room temperature in a vacuum desiccator. PDMS was cured at 85°C for 2 h. Cured PDMS was peeled from the wafers. Media inlets and outlets were punched with a 6 mm-diameter biopsy punch and then extra PDMS was removed with a razor blade. Prepared devices and standard microscope slides (Electron Microscopy Sciences) were sonicated in ethanol, cleaned in water, and blown dry with filtered air. Devices and slides were then activated by exposure to oxygen plasma (Harrick Plasma) for 2
min 15 s at 18W to render the surfaces hydrophilic. Activated devices were attached to activated glass slides, then coated with a 20 μg/mL solution of rat tail collagen-I (Gibco) in phosphate buffered saline (PBS, Life Technologies/Gibco) without magnesium or calcium for 1 hour at 37°C.

4.2.3 Cell seeding, imaging, and inhibitors used. Cells were passed and resuspended in media to a concentration of 1-2 x 10⁶ cell/mL. All solution was removed from microfluidic device wells by vacuum aspiration. 20 μL of cell suspension was added to the cell inlet and the incubated for 5 min at 37°C and 5% CO₂. Cell suspension was then moved from the cell inlet to the cell outlet to seed the other side of the microchannels and incubated for another 5 min. After appropriate treatment, cell behavior was captured through a 10x/0.30 numerical aperture Ph1 objective lens in 10 min intervals for 14 h by a Digital Sight Qi1Mc camera mounted on a Eclipse Ti-E Inverted microscope (Nikon) equipped with a stage-top incubator (Tokai Hit Co., Shizuoka, Japan) maintained at 37°C with 5% CO₂ and 100% humidity.

For select experiments, cells were treated with the following pharmacological inhibitors (purchased from Sigma Aldrich unless otherwise stated): 2-APB (Calbiochem, 100065), FTY-720, Thapsigargin, PD98059 (Cell Signaling Technology, 9900), GSK2193874, BAPTA, ruthenium red, blebbistatin, Y27632, ML141 (Santa Cruz Biotechnology), Ionomycin.

4.2.4 Generation of shear flow via hydrostatic pressure gradient. Fibroblast cells were seeded in the microfluidic devices. Once firmly adhered to the collagen-I-coated seeding channel, 50 μL of media was placed in each of the 6 wells of the microfluidic device, such that the hydrostatic pressure in the device was equal everywhere and the media was at rest (static). At this point, the devices were incubated at 37°C and 5% CO₂ for 2-3 hours, until cells had entered and migrated to the center of most of the microchannels. Then, the media was aspirated from all wells, and 167 μL of fresh media was placed in the inlet wells to the seeding and media channels, such that a
hydrostatic gradient was generated across these larger channels, while pressure across the perpendicular microchannels was balanced.

Aqueous fluids in microfluidic devices can generally be considered incompressible, and thus their flow can be modeled by the Navier-Stokes equation for Newtonian fluids with no body forces (265, 266). For laminar fluid flow, the fluid flow rate resulting from the pressure gradient can be modeled in the channels as (267):

\[ \Delta p = \frac{12 \mu L Q}{WH^3} \left[ \frac{1}{1 - 0.63 \frac{H}{W} \tanh \left( \frac{1.57 W}{H} \right)} \right] \]  

where \( \mu \) is the fluid viscosity (approximated by the viscosity of water), \( L \) is the length of the channel, \( Q \) is the volumetric flow rate, \( W \) is the channel width, and \( H \) is its height. While the exact solution for flow in rectangular ducts requires expansion of a Taylor series, a good approximation of the wall shear stress (\( \tau_w \)) on the lower surface in front of the microchannel exits (where migrating cells contact fluid flow) exists based on the low aspect ratio (\( \alpha \), width over height) of our microfluidic channels (0.075) the assumptions of steady-state, low Reynolds number (\( Re \ll 1 \) (268, 269):

\[ \tau_w = \frac{\delta \mu Q}{WH^2} \left( \frac{m+1}{m} \right) (n + 1) \]  

where \( m \) and \( n \) are empirically-derived constants with \( m = 1.7 + 0.5 \alpha^{-1.4} \), where \( \alpha \) is the aspect ratio as defined above and \( n = 2 \) for \( \alpha < 1/3 \). Using these equations, we verified that the Reynold’s number based on the area-averaged flow velocity is ~0.03 (\( Re \ll 1 \)).

Fluid flow from the inlet well to the outlet well reduces the pressure gradient over time. Equating the change in fluid volume in the inlet well rate over time to the fluid flow rate:

\[ -a \frac{dh}{dt} = \frac{\rho g h W H^3}{L 12 \mu} \left[ 1 - 0.63 \frac{H}{W} \tanh \left( \frac{1.57 W}{H} \right) \right] \]  

where \( a \) is the cross-sectional area of the inlet/outlet wells and \( h \) is the difference in height between these wells. Rearrangement and application of integration limits leads to:

\[ -a \int_{h_i}^{h_f} \frac{dh}{h} = \frac{\rho g W H^3}{L 12 \mu} \left[ 1 - 0.63 \frac{H}{W} \tanh \left( \frac{1.57 W}{H} \right) \right] \int_0^t dt \]
which can be solved for the final form:

$$h_f = h_1 10^{-0.63gWh^2 \left[ 1 - 0.036 \tanh \left( 1.57 \frac{W}{H} \right) \right] t}$$

(5)

Equation (5) can be used to determine the change in pressure gradient as a function of time. Updating of the pressure gradient driving the flow rate in equations (1) and (2) reveals the hydrostatically driven shear stress in our system as a function of time:

Figure 4-1: Relationship between hydrostatic pressure gradient-driven shear stress and time. Calculations made for microfluidic device dimensions using equation (5) to determine the changing driving force for fluid flow. 75% of initial shear rate maintained until 1.6 h (dotted line) and 50% of shear rate maintained until 3.7 h (dashed line).

4.2.5 Fluo-4 Direct. Fluo-4 direct working solution was prepared to 2X according to manufacturer instructions (Fluo-4 Direct Calcium Assay Kit, Sigma Aldrich). Working solution was diluted to 1X with cell media, and cells already seeded in microfluidic devices were treated with this 1X solution for 1 hour at 37°C and 5% CO₂. Fluo-4 direct solution was then removed from the cells and replaced with 167 µL regular media in every well then incubated for 1 hour to allow the cells to come to rest after any physical stimulation from the media change. Microfluidic devices were then placed on an A1 confocal microscope (Nikon) equipped with a stage-top incubator and cage (Tokai Hit Co., Shizuoka, Japan) maintained at 37°C with 5% CO₂ and 100% humidity. Cells were imaged in 6 s intervals for 5 min under static conditions. Flow was induced
by aspirating the media from the two outlet wells, and then imaging was resumed. For inhibitor experiments, the drug or vehicle control was introduced during the 1 h incubation period prior to imaging. Fluorescence intensity of the collected images was analyzed using ImageJ (National Institutes of Health). Fluorescence intensity of individual cells was quantified by outlining the cell at each time point using polygonal regions of interest.

4.2.6 shRNA design and cloning and LifeAct Imaging. Silencing RNA sequences to relevant genes were retrieved from the Gene Perturbation Platform (Broad Institute). The targeting sequences are:

- Scramble Control: (GCACTACCAGAGCTAACTCAGATAGTACT)
- human TRPC1 sequence-1 (TTCTCGTGAATTGGAAGTTAT)
- human TRPC1 sequence-2 (GCCACCTGTAAGAAGATAAT)
- human TRPC6 sequence-1 (GTCCACTTGAAGCCATATTAT)
- human TRPC6 sequence-2 (CGCTCCACAAGCCTATCTATA)
- human TRPM7 sequence-1 (CAGCAGAGCCCGATATTATTT)
- human TRPM7 sequence-2 (GCAGATCTGCTAGCGTATATT)
- human Cdc42 sequence-1 (CGGAATATGTACCGACTGTTT)
- human Cdc42 sequence-2 (CCTGATATCCTCACACAACC)

Sense and antisense silencing sequences were added on either end of a short hairpin loop and completed forward and reverse sequences were ordered as custom DNA oligomers (Life Technologies).

To generate shRNA lentiviral plasmids, we cloned the targeting oligomer sequences or non-targeting scramble control into the pLVTHM lentiviral plasmid (270) (a gift from Dider Trono, plasmid # 12247). Cdc42 targeting sequences and scramble control sequences were cloned into the pLKO.1 lentiviral plasmid (a gift from David Root, plasmid # 10878). Briefly, forward
and reverse sequences were annealed by mixing and incubating on a thermocycler for 3 min at 95°C, 2 min at 72°C, 2 min at 37°C, 2 min at 25°C. Annealed oligos and 1 µg plasmid were digested with MluI and ClaI (pLVTHM) or BshTI and EcoRI (pLKO.1) restriction enzymes (New England Biolabs) in separate reactions according to the manufacturer’s instructions. Digested products were run on a 0.8 % agarose dye for 45 min at constant 115 V. Digested fragments were excised with clean razor blades and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). 1:1 ratios of digested oligos and plasmid were incubated with T4 DNA ligase (New England Biolabs) according to the manufacturer’s protocol. StBl3 competent bacteria (Invitrogen) were transformed with the ligated products and streaked on ampicillin agar plates then incubated overnight at 37°C. Individual surviving colonies were picked and expanded for 12 h in 3 mL Luria Bertani broth (Quality Biological) containing 100 µg/mL ampicillin. Expanded plasmids were isolated using QIAprep Spin MiniPrep Kits (Qiagen). Sequence integrity and orientation was verified by Sanger Sequencing (JHU Genetic Resources Core Facility).

The pLVTHM, pLKO.1, pLenti.PGK.LifeAct-GFP.W (gift from Rusty Lansford, plasmid # 51010), RhoA2G FRET biosensor (gift from Olivier Pertz, plasmid # 40176, #40179), psPAX2 (gift from Didier Trono, plasmid # 12260), pMD2.G (gift from Didier Trono, plasmid # 12259) plasmids were purchased from Addgene.

To produce lentivirus, 293T/17 cells were co-transfected with psPAX2, pMD2.G, and the appropriate lentiviral plasmid. Lentivirus was collected after 48 h after transfection, filtered through 0.45 µm filters (Fisher Scientific) and concentrated by centrifugation (50,000 x g for 2 h at 4°C). Cells were transduced for 24 h with media containing lentiviral particles. Cells transduced with pLKO.1 were selected with puromycin (0.5 µg/mL, Gibco) 48 h after transduction and maintained with this concentration. Migration experiments with cells transduced with pLVTHM were also imaged using 488nm laser and only cells expressing green fluorescent protein (encoded on pLVTHM) were analyzed.
4.2.7 qPCR. Standard qPCR techniques were applied as described previously (271) using the following primers:

TRPM7: F-(5’-GGAGATGCCCTCAAAGAACA) and R-(5’-TGCTCAGGGGGTTCAATAAG)
TRPC1: F-(5’-GATGTGCTTGGAGAAATGC) and R-(5’-AATGACAGGTGCAACATCCA)
TRPC6: F-(5’-AGACAATGCGGGTCAAAGTTC) and R-(5’-TCCCAGAAAAATGGTGAGG)
Piezo1: F-(5’-TTCTGCTGTACCAGTACCT) and R-(5’-AGGTACAGCCACTTGATGAG)
TRPV2: F-(5’-CAAACCGATTTGACCGAGAT) and R-(5’-GTTCAGCACAGCCTTCATCA)

4.2.8 LifeAct Imaging. Fibroblast cells were treated with LifeAct-GFP lentivirus generated as described above. Cells were seeded in microfluidic devices and imaged through a 20x/0.4 numerical aperture Ph1 lens using an Eclipse Ti-E Inverted microscope (Nikon) equipped with an Intensilight C-HGFIE arc lamp (Nikon) and the appropriate excitation and emission filters (Nikon).

4.2.9 Immunofluorescence. Cells were fixed with 4% formaldehyde solution (ThermoFisher Scientific), permeabilized with 1% Triton® X-100 (Sigma Aldrich), blocked against nonspecific adhesion with 2% bovine serum albumin (Sigma Aldrich) and 1% goat serum (ThermoFisher Scientific), immunostained for target proteins, and then imaged on an A1 confocal microscope (Nikon). Primary antibodies were administered at manufacturer recommended concentration: anti-P-MLC (Ser19, 3671, Cell Signaling Technology), Rhodamine phalloidin (ThermoFisher Scientific). Quantification of P-MLC and actin stress fiber density performed as described in (16).

4.2.10 Fluorescence lifetime imaging microscopy (FLIM). For measurements of active RhoA, cells stably transduced with pLentiRhoA2G were imaged using a Zeiss LSM 780 microscope fitted to a PicoQuant FLIM system consisting of the PicoHarp 300 time-correlated single photon
counting (TCSPC) module, *Sepia II* laser control module and two hybrid PMA-04 detectors. A *PeCon* environment chamber was used to maintain the cells at 37°C and 5% CO₂ during imaging.

The FLIM data was acquired with a Apochromat 40x/1.1W Corr 27 lens (Zeiss), using 440nm diode excitation laser pulsed at 32MHz and reflected to the sample through a 80/20 mirror (Zeiss). Emission light below 488nm was collected at the PicoQuant PMA-04 hybrid detector by a dichroic mirror after it passed through a transparent plate and optic fiber. Images were acquired at a single scan of 1024 X 1024 pixel with a pinhole size between 3 µm and 1.5 µm z-sections (to limit the emission photon count rate below 10% of the laser excitation rate). TCSPC data was acquired in 8ps time bins within 31.25ns time window.

The FLIM data was processed with *SymPhoTime 64* (PicoQuant) software by using a custom written script for the calculation of the internal response function (IRF) from 100 data points with no smoothing. A constant threshold was applied to eliminate fluorescent signal from outside the cell area and the data was binned to obtain 600-1000 photons per binned pixel. Then, a 3-exponential reconvolution was used to fit the fluorescence decays into every binned pixel. As the RhoA FRET sensor was mostly excluded from the nucleus resulting in an inconsistent and weak photon output, the nuclear pixels were manually excluded by drawing a free ROI around the nucleus during the final segmentation. Next, using the software we calculated the amplitude-weighted fluorescence lifetime average of the total cell.

**4.2.11 Western Blotting.** Western blots were performed as previously described (272, 273) using NuPage 4-12% Bis-Tris gels and the following antibodies (at the manufacturer-recommended concentrations): anti-Cdc42 (rabbit, clone 11A11, Cell Signaling Technology 2466) with β-actin as a loading control (Purified Mouse Anti-Actin Ab-5, BD 612656). Secondary antibodies (used at 1:1000 dilution): anti-mouse IgG HRP-linked antibody (Cell Signaling Technologies), anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies).
4.2.12 Survival analysis. To examine the role of TRPM7 in breast and liver cancer metastasis, patient overall survival (OS) time and gene expression data were downloaded from the KM-plotter database (274), which combines datasets published on GEO, EGA and TCGA. Patients were split into 2 groups based on their expression levels of TRPM7. The expression cutoff producing the greatest separation of OS between the 2 groups was used to generate Kaplan-Meier survival curves and statistical results. Importantly, comparing the lowest and highest tertiles or quartiles produced similar results.

4.2.13 Statistical analysis. Unless otherwise specified, data represent the mean ± standard deviation of ≥ 3 independent experiments. The D’Agostino-Pearson omnibus normality test was used to determine if data are normally distributed. Data sets with gaussian distributions were compared using an unpaired student’s t test (two-tailed) or ANOVA followed by multiple comparisons testing. Statistical significance was defined as $p < 0.05$. Calculations were performed using GraphPad Prism 7 (GraphPad Software).

4.3 Results

4.3.1 Migrating fibroblasts sense shear flow and reverse migration direction. In the absence of a chemotactic gradient, dermal fibroblasts spontaneously migrate from a 2D-like seeding region through microchannels. Upon reaching the end of the microchannel, typically over 70% of these cells will exit into the 2D-like region on the far side under static conditions (Fig. 4-2A). Interestingly, when shear stress of 0.5 dyne/cm$^2$ is created in both 2D-like regions by hydrostatic pressure-driven fluid flow, the majority (80%) of cells migrating in the microchannels that reach the end reverse their migration direction and remain within the microchannels (Fig. 4-2A). This phenomenon was observed regardless of the width of the microchannels (Fig. 4-2A), including in smaller microchannels with a width of 6 µm or 3 µm (data not shown); however, because the
fibroblast cells enter these narrower microchannels at a low late, we focused on the 10 µm wide microchannels, which impose physical confinement on cells of this size. In line with these results, newborn human foreskin fibroblasts (NuFFs) and Chinese hamster ovary (CHO) cells also are sensitive to shear flow at this level, and reverse direction at the end of microchannels in flow, but not static conditions (Fig. 4-2B-C).
Figure 4-2: Migrating fibroblast cells sense shear flow and reverse their migration direction. Primary dermal fibroblast cell migration was observed in collagen-I-coated microchannels of width, $W = 10-50 \, \mu m$, and height, $H = 10 \, \mu m$. Parallel to the microchannels are larger 2D-like channels ($W = 400 \, \mu m$ and $H = 50 \, \mu m$) where fluid flow is controlled. (A) Percentage of primary human dermal fibroblast (B) newborn human foreskin fibroblast or (C) Chinese hamster ovary migrating cells that reach the end of the microchannels and then reverse their migration direction to remain in the microchannel when the media in the larger channels is either static or flowing. Data represent the mean and SD from $\geq 3$ independent experiments. Data points represent the percentage from each individual experiment. *** $P < 0.001$ relative to same channel dimension and static conditions, Two-way ANOVA followed by Sidak’s multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, Student’s $t$-test.
4.3.2 Extracellular calcium influx controls cell migration direction. Previous work has demonstrated that calcium signaling plays a role in controlling persistent cell migration direction, chemotaxis, and in response to physical force (75, 102, 275). To elucidate the role of calcium signaling in shear sensation, intracellular calcium was visualized in migrating fibroblasts using Fluo-4 Direct. The arrival of the cell at the end of the microchannel typically coincided with a sharp increase in Fluo-4 Direct fluorescence, indicating increased intracellular concentration, followed by a reversal of their migration direction (Fig. 4-3A). Due to the relatively low throughput of this measurement of calcium activity within cells confined in microchannels, we examined larger populations of cells in the 2D-like seeding channels of the microfluidic devices (2D). Cells were imaged for 1 minute under static conditions and then exposed to hydrostatic pressure-driven fluid shear flow by removing the media from the outlet well, which caused a sharp spike in fluorescence from Fluo-4 Direct ($\Delta F/F_o$, where $\Delta F = F - F_o$, $F$ = fluorescence at a given time point, and $F_o$ = average fluorescence intensity under static conditions) (Fig. 4-3B-C). Cellular calcium signaling is disrupted at several levels by 2-aminoethoxydiphenyl borate (2-APB), which inhibits the entry of extracellular calcium into the cell through many transient receptor potential (TRP) channels and antagonizes store-operated calcium release by inhibiting its mediator, inositol triphosphate receptor (IP$_3$R) (276, 277). Fibroblast cells treated with 2-APB in 2D had significantly dampened calcium increase when exposed to shear stress (Fig. 4-3B-C). Application of 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride (FTY720), a specific inhibitor of the transient receptor potential melastatin 7 (TRPM7) channel (278), also significantly lowered intracellular Fluo-4 Direct fluorescence in response to shear flow (Fig. 4-3B-C). To determine if these changes in 2D calcium response to shear flow affect the ability of cells in microchannels to sense and respond to shear flow, migrating fibroblasts were treated with 2-APB (Fig. 4-3D) or FTY720 (Fig. 4-3E), and both interventions allowed fibroblasts to exit the microchannels in the presence of shear stress. A general TRP channel inhibitor, ruthenium red (RR) (279, 280), enabled fibroblasts to exit into shear flow (Fig. 4-4A).
Figure 4-3: Extracellular calcium signals to reverse cell migration direction. (A) Fluo-4 direct fluorescence to visualize intracellular calcium in a migrating dermal fibroblast cell as it...
reaches the end of a microchannel where shear flow is present. Time stamp is in h:min, scale bar is 10 µm. (B) Fluo-4 Direct fluorescence to visualize intracellular calcium in dermal fibroblast cells in a 2D-like channel imaged every 6 seconds for 1 min under static conditions followed by exposure to shear flow. Flow 0s corresponds to images taken quickly after flow was induced. Scale bars are 50 µm. (C) Quantification of Fluo-4 Direct fluorescence from B, ΔF/Fo, where ΔF = F-Fo, F = fluorescence at a given time point, and Fo = average fluorescence intensity under static conditions. Data represent mean and SE for n > 25 cells pooled from 3 independent experiments. * P < 0.05, Two-way ANOVA followed by Tukey’s multiple comparisons test. (D) Percentage of cells that reverse migration direction when treated with 2-APB (100 µM), (E) FTY-720 (2 µM), or (F) BAPTA or a vehicle control (VC). (G) Percentage of cells that reverse migration direction under static or flow conditions after shRNA-mediated knockdown of TRPC1, TRPC6, or TRPM7 compared to cells treated with a scramble control (SC) sequence. Data represent mean and SD from n ≥ 3 independent experiments. Data points represent the percentage from each individual experiment. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, One-way ANOVA followed by Tukey’s multiple comparisons test.
Figure 4-4: Extracellular calcium signals to reverse cell migration direction. (A) Percentage of cells that reverse migration direction when treated with ruthenium red (RR) (10 µM) or a vehicle control. Data represent mean and SD from 3 independent experiments. Data points represent the percentage value from individual experiments. * $P < 0.05$, One-way ANOVA followed by Tukey’s multiple comparisons test. (B) Quantitative PCR measurement of mRNA transcript levels in cells treated with shRNA sequences to TRPC1, TRPC6, TRPM7, or a noncoding scramble control sequence (SC). Expression of each gene was normalized to and statistically compared to that of the SC. Data represent mean and SD from 3 independent experiments ** $P < 0.01$, **** $P < 0.0001$, One-way ANOVA followed by Tukey’s multiple comparisons test.
These results suggest that entry of extracellular calcium into the cell in response to shear flow may be a key step in a fibroblasts cell’s ability to sense shear flow and reverse migration direction. Indeed, chelation of extracellular calcium with 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraaetic acid (BAPTA) (281) allowed migrating fibroblasts to exit microchannels into shear flow at equal rates to that of cells exiting into static conditions (Fig. 4-3F). To identify if a specific ion channel is responsible for allowing entry of extracellular calcium in the presence of shear flow, short hairpin RNAs (shRNAs) were designed to specifically knockdown TRP channels targeted by the panel of inhibitors used, including TRPC1, TRPC6, and TRPM7. Knockdown of each gene was confirmed by quantitative polymerase chain reaction (qPCR) (Fig. 4-4B). Stable knockdown of TRPC1 and TRPC6 did not affect fibroblast cells’ ability to sense shear flow and reverse their migration direction. In contrast, TRPM7 knockdown cells exited the microchannels at a similar rate into static or flow conditions, suggesting that they could not sense the difference between these two conditions.

4.3.3 Shear flow activates myosin contractility. To understand the mechanism leading cells that contact shear flow to reverse their migration direction, we investigated the dynamics of the actin cytoskeleton of migrating fibroblasts by observing LifeAct-GFP. These fibroblasts typically migrate through the microchannels with protrusion-based morphologies (> 90% of cells), suggesting an actin-polymerization driven migration mechanism (Fig. 4-5A-B). Interestingly, upon reaching the end of microchannels and sensing shear flow, roughly half of the cells that reverse their migration direction switch to a bleb-based migration mode (Fig. 4-5A-B) exhibited by strong polarization of the actin cytoskeleton to the cell leading and trailing edges and the formation of small round membrane protrusions – termed blebs. Fibroblast cells that exit the microchannels into static conditions in the 2D-like channel at the end of the microchannels spread fully (> 90% of cells), whereas when flow is present, only half of the exiting cells are able to spread (Fig. 4-5C), and many maintain a round morphology or have a bleb-based morphology.
The blebbing and rounded morphologies induced by shear flow suggest increased actomyosin contractility (8, 282). Shear stress is known to induce mechanosensitive calcium signaling to increase cellular contractility (102, 283). Along these lines, fibroblast cells in 2D had increased phosphorylation of myosin light chain 2 (Ser19) and actin stress fiber density after 5-minute exposure to shear stress compared to cells maintained under static conditions (Fig. 4-5D-F), demonstrating that even relatively short exposure to shear stress increases cellular contractility.
Figure 4-5: Shear flow activates myosin contractility. (A) Representative images of a migrating fibroblast expressing LifeAct-GFP imaged by epifluorescence at 20x magnification. Shear flow is present perpendicular to the microchannel exits. Open triangles mark cell protrusions. Closed triangles mark cell blebs. Time stamp is in hours. (B) Percentage of fibroblast cells migrating through microchannels with protrusive or bleb-based morphology (with flow present at the exits). Left bar, all cells migrating in the microchannel. Right bar, only cells that reach the end of the microchannel and reverse their migration direction. (C) Percentage of fibroblast cells exhibiting a protrusive, blebbing, or rounded morphology after exiting the microchannel into a 2D-like area under static (left) or flow (right) conditions. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$. (D) Immunofluorescence images showing MLC phosphorylation and actin filaments. (E) Phalloidin staining showing actin filament density. (F) MLC phosphorylation density. (G) Representative images showing cell morphology under static and flow conditions. (H) Ushkoi and Ushkoi-2 density measurements. (I) Cells that reach channel exit and reverse direction percentage. (J) Cells that reach channel exit and reverse direction percentage with VC and Blebbistatin treatment. (K) Cells that reach channel exit and reverse direction percentage with Y27632 treatment.
Two-way ANOVA followed by Sidak’s multiple comparisons test. Data represent mean and SD from 3 independent experiments. Data points represent percentage values of protrusive cells from individual experiments. (D) Representative confocal images of cells in 2D under static conditions or exposed to shear flow for 5 min and then fixed and immunostained for P-MLC and stained with phalloidin (actin) and Hoechst 33342 (nucleus). (E) Quantification of P-MLC fluorescence or (F) Actin stress fiber density. Data points represent values for individual cells pooled from 3 independent experiments. ** P < 0.01, *** P < 0.001, Student’s t-test. (G) Representative heatmaps of RhoA FRET fluorescence lifetime in fibroblast cells under static or flow conditions. Heat map is color coded to corresponding values of fluorescence lifetime (ns) at each pixel. (H) Quantification of FLIM-FRET lifetime from images in G. Reduced fluorescent lifetime corresponds to increased RhoA activity. Data points represent the average fluorescent lifetime over a whole cell, pooled from 1 independent experiment. **** P < 0.0001, Student’s t-test. (I) Percentage of cells that reverse migration direction when treated with blebbistatin (50 µM) or (J) Y27632 (10 µM) or a vehicle control (VC). Data represent mean and SD from 3 independent experiments. Data points represent the percentage value from individual experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, One-way ANOVA followed by Tukey’s multiple comparisons test.
The small GTPase protein, RhoA, is also known to be activated by mechanical force (284) and has been extensively studied due to its role in cellular contractility and migration (285). Comparison of RhoA activity between 2D cells in static versus flow conditions was made by fluorescence lifetime imaging microscopy (FLIM) measurements of Förster resonance energy transfer (FRET) and revealed that shear stress activated RhoA, as demonstrated by the decreased fluorescence lifetime in the flow condition (Fig. 4-5G-H). Finally, to determine if contractility is required for migrating fibroblasts to sense shear flow and reverse their migration direction, we inhibited myosin contractility or its upstream regulator, Rho-associated protein kinase (ROCK), with blebbistatin or Y27632, respectively. Interestingly, inhibition of myosin reduced the cells’ ability to reverse direction after sensing shear flow (Fig. 4-5I) but did not reduce this behavior to the level of cells in static conditions, indicating some remaining mechanosensitivity. ROCK inhibition reduced the rate of cell reversal to that of the static condition (Fig. 4-5J).

4.3.4 Cell polarity reorganization is required to reverse migration direction. Persistent cell migration is characterized by distinct protein activity at the cell leading and trailing edges (286, 287). Disruption of these cell front and cell rear signals leads to changes in the cell migration direction (102, 288, 289). The Rho GTPase, Cdc42, is active at the leading edge of a cell, and its optogenetic activation on any edge of the cell leads to persistent migration in that direction in mouse RAW 264.7 macrophage-like cells, mouse embryonic fibroblasts (MEFs), and HeLa human cervical cancer cells (290-292). Activation of Cdc42 activity to determine the cell leading edge leads to myosin-II localization to the cell trailing edge (290), and this myosin activity is necessary for persistent directional migration even during sustained Cdc42 activation at one edge of the cell (291). Based on the localized activity of Cdc42 at the cell leading edge and myosin-II at the trailing edge, we hypothesized that when migrating cells reach the end of the microchannel and experience force from shear flow, myosin-II is activated at the leading edge of the cell, disrupting the cells’ established polarity. The cells are then forced to re-establish cell polarity,
leading some cells to reverse their migration direction in accordance with the locally activated myosin-II, and some to recover their initial polarity and exit the microchannel into the 2D space. According to this hypothesis, disruption of Cdc42 should inhibit the cells’ ability to re-establish polarity and reverse cell migration direction after experiencing shear stress. Inhibition of Cdc42 with the inhibitor ML141 (Fig. 4-6A) or stable knockdown of Cdc42 with either of two shRNA sequences (Fig. 4-6B-D) prevented fibroblast cells from reversing their migration direction when they encountered shear flow. This suggests that re-establishment of cell polarity signaling downstream of Cdc42 is required to respond to this stimulus.
**Figure 4-6: Cdc42 activity required to reverse migration direction.** (A) Percentage of cells that reverse migration direction at the end of the microchannels when treated with ML141 (10 µM) or a vehicle control (VC). (B) Representative western blot of fibroblast cells treated with shRNA sequences against Cdc42 or a noncoding scramble control (SC) sequence. Actin was used as a loading control. Cell lysis and blotting was repeated 3 independent times. (C-D) Percentage of SC or Cdc42-KD cells that reverse migration direction at the end of the microchannels. Data represent mean and SD from ≥ 2 independent experiments. Data points represent the percentage value from individual experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, NS P ≥ 0.05, One-way ANOVA followed by Tukey’s multiple comparisons test.
4.3.5 Fibrosarcoma cells have altered ion channel expression and reduced ability to respond to calcium stimulus. During cancer metastasis, migrating tumor cells encounter a range of physical stimuli from the architecture of the extracellular matrix, local tissue stiffness and compression, and shear stresses in the circulatory system (5, 6, 223). Based on the fibroblasts’ aversion to exiting microchannels into shear flow, we hypothesized that cancers arising from this cell type, such as fibrosarcoma, would have to overcome this mechanism in order to intravasate into the blood stream, where shear stress would otherwise stimulate calcium influx that could trigger the cells to reverse their migration direction and not exit into the bloodstream. Indeed, reduced expression of TRPM7 corresponds to reduced overall survival times in breast and liver cancer according to Kaplan-Meier survival analysis (Fig. 4-7A-B), suggesting that TRPM7 may inhibit cancer progression in these cancers.
Figure 4-7: Reduced TRPM7 expression correlates to poor overall survival in breast and liver cancer patients. (A) Breast cancer and (B) and liver cancer patient overall survival (OS) time and gene expression data were downloaded from the KM-plotter database, which combines datasets published on GEO, EGA and TCGA. Patients from each cancer were split into 2 groups based on their expression level of TRPM7 and the cutoff producing the greatest separation of OS between the high- and low-expressing groups was used. Kaplan-Meier survival analysis revealed that patients expressing low levels of TRPM7 had significantly decreased OS.
In stark contrast to fibroblast cells, migrating HT-1080 fibrosarcoma cells consistently exit the microchannels into both static and shear flow conditions (Fig. 4-8A). We hypothesized that the HT-1080 cells may have altered expression of mechanosensitive ion channels. Indeed, qPCR revealed that the HT-1080 cells have reduced expression of TRPM7, TRPC1, and PIEZO1 but increased expression of TRPV2 compared to the fibroblasts (Fig. 4-8B). As TRPM7 is required for the fibroblasts to respond to shear flow by reversing their migration direction, it is possible that the downregulation of this channel is responsible for the HT-1080 cells’ ability to exit the microchannels into shear flow. To investigate if calcium signaling downstream of ion channels is sufficient to cause these cell types of reverse their migration direction, we filled the seeding channel and microchannels with control media, and added media containing the ionophore, ionomycin, to the exit channel (Fig. 4-8C), such that migrating cells that reach the end of the microchannels will have increased intracellular calcium under static conditions. The presence of ionomycin at the end of the microchannels caused fibroblast cells that arrived there to reverse their migration direction in a dose-dependent manner, up to an average of 42% of cells at 10 µM ionomycin (Fig. 4-8D). As this percentage is similar to that of shear-induced cell reversal, this data suggests that calcium signaling is sufficient to cause fibroblast cells to reverse their migration direction. HT-1080 cells also showed a dose-dependent increase in the percentage of cells that reversed their migration direction upon reaching the ionomycin at the channel end, but on average only 15% of these cells responded to this stimulus (Fig. 4-8E). This suggests that a stronger stimulus, or the combination of other stimuli is required to render these cells sensitive to shear flow.
Figure 4-8: Fibrosarcoma cells do not demonstrate sensitivity to low levels of shear stress.  

(A) Percentage of HT-1080 fibrosarcoma or normal fibroblast cells that reverse migration direction at the end of the microchannels for static or flow conditions. Data represent mean and SD from 5 independent experiments. Data points represent the percentage value from individual experiments. **** P < 0.0001, One-way ANOVA followed by Tukey’s multiple comparisons test. (B) Relative mRNA expression of HT-1080 and fibroblast cells assessed by qPCR. Data represent mean and SD from 3 independent experiments. * P < 0.05, One-way ANOVA followed by Tukey’s multiple comparisons test. (C) Schematic of microfluidic device after filling with regular media, and replacement of the media in the channel opposite the seeding channel with media containing ionomycin (1-10 µM) or a vehicle control. (D) Percentage of fibroblast or (E) HT-1080 cells that reverse migration direction at the end of the microchannels when the media in the channel opposite the seeding channel is treated with ionomycin or a vehicle control as shown.
in C. Data represent the mean and SD from at least 3 independent experiments. Data points represent the percentage from individual experiments. * $P < 0.05$ relative to VC, ** $P < 0.01$ relative to VC, One-way ANOVA followed by Tukey’s multiple comparisons test.
4.4 Discussion

To study the interplay between topographically-directed cell migration and entry into a channel with shear stress, we used an engineered *in vitro* model of cell migration in longitudinal tracks followed by entry into a larger channel with shear flow. Fibroblast cells (including primary human dermal fibroblasts, newborn human foreskin fibroblasts, and fibroblast-like CHO cells) migrating in longitudinal tracks that intersect fluid flow at relatively low shear stress (0.5 dyne/cm²) frequently reversed their migration direction away from the shear stress. In sharp contrast, human HT-1080 fibrosarcoma cells were not averse to shear flow at this level and consistently exited the microchannels into the larger orthogonal channel under both static and shear conditions. Fibroblasts sensed shear flow via entry of extracellular calcium to the cell, followed by calcium-activated calcium release from the endoplasmic reticulum. Calcium entry appeared to be governed by the mechanosensitive ion channel, TRPM7. FLIM-FRET and immunofluorescence revealed that shear flow induced RhoA activity, cellular contractility, and actin polymerization. Pharmacological inhibition of myosin contractility and ROCK reduced fibroblast shear sensitivity. Successful change in migration direction upon exposure to shear flow required Cdc42 activity, as inhibition or knockdown of Cdc42 prevented this behavior. Migrating cancer cells were less sensitive to low levels of shear flow than fibroblasts. This may in part be due to their downregulation of mechanosensitive ion channels, including TRPM7. However, sudden increase of calcium in cancer cells only caused about 15% of these cells to reverse their direction, compared to about 50% of fibroblast cells. This suggests that fibrosarcoma cells may have reduced sensitivity to calcium signaling, such as a deficiency in RhoA activation. Altogether, fibroblast and cancer cells displayed different levels of sensitivity to low levels of shear stress and calcium activation, which may promote cancer cell dissemination.

The effect of shear stress on fibroblast migration is understudied (293). Laminar and pulsatile shear stress cause fibroblasts on 2D substrates to align themselves in line with the
direction of flow (294-296). In contrast, exposure of dermal fibroblasts embedded in 3D collagen gels to shear stresses of 0.3-1.1 dyne/cm² caused alignment perpendicular to the direction of flow (168), highlighting the importance of studying cellular behavior in physiologically relevant environments. Human dermal fibroblasts plated in 2D migrate downstream, away from the source of flow in response to shear stresses from 5-20 dyne/cm² (297). Exposure of rat adventitial fibroblasts plated on transwell inserts to 20 dyne/cm², but not 1 dyne/cm² using a radial disk apparatus enhanced their migration through the inserts (perpendicular to the flow direction) while suppressing that of myofibroblasts (298). These results are along the lines of our findings that shear stress coordinates fibroblast migration, although at different shear thresholds; it is possible that the adventitial fibroblasts are conditioned to shear stress due to their proximity to circulatory vessels (293). Exposure of the same rat adventitial fibroblasts to shear stress from 0.05-0.36 dyne/cm² while embedded in 3D collagen gels caused upregulation of MMP-1 and protease-dependent migration downstream within the gel. In all, these results are in agreement with our findings that even low levels of shear stress regulate fibroblast migration, and that these cells tend to move away from the source of flow in several environments. These studies do not uncover the specific mechanism by which fibroblasts sensed or responded to shear flow. The cellular process of converting physical force to biochemical signals is known as mechanotransduction. Cells sense physical force through a variety of mechanisms, including transmembrane receptors, mechanosensitive ion channels, their glycocalyx, and integrins (293, 299). Recent work has also implicated the nucleus as an important mechanosensor (300, 301). Other work by Wei et al demonstrated the entry of extracellular calcium through TRPM7 in response to the application of external physical force to fibroblast cells, which agrees with our findings (75, 102). Wei et al described the activity of local calcium flickers at the leading edge to guide cell migration, in the presence of a global calcium gradient with increased calcium concentrations localized to the rear of the cell (75, 102). Although highly localized calcium flickers were not observed during our experiments, it is possible that the increased total intracellular calcium in response to shear flow
disrupted the front-to-back calcium gradient, thereby preventing sustained forward migration (154).

Shear stress has also been demonstrated to modulate cancer cell behavior. Several mechanotransduction pathways have been implicated in the response of cancer cells to fluid shear stress. In prostate cancer cells exposed to low levels of shear stress (0.05 dyne/cm$^2$) in the lumen of a biomimetic vessel, integrin mechanosensing activated a ROCK-LIMK-cofilin signaling axis to promote actin integrity, potentially transmitted force to the nucleus, and induced nuclear translocation of yes-associated protein 1 (YAP1) (302). In this study, shear stress $\leq 5$ dyne/cm$^2$ enhanced cancer cell migration, while higher shear stresses inhibited migration (302). In MDA-MB-231 breast cancer cells, fluid shear stress (1.8 dyne/cm$^2$) rapidly activated PI3K via $\alpha_\beta_3$ integrin and caveolin-1, an intracellular plasma membrane-associated protein, leading to enhanced wound healing and invadopodia formation (303, 304). Exposure of human renal carcinoma cells to fluid shear stress (0.8 dyne/cm$^2$) caused glycocalyx-mediated upregulation of MMP-1 and -2, Cd44, $\alpha_3$ integrin, and caveolin, leading to enhanced transwell invasion following exposure to flow (305). Shear rates during parts of circulation, such as in the heart, are orders of magnitude higher, and can induce cell apoptosis (185, 186). Circulating tumor cells (CTCs) from breast (MDA-MB-231) or prostate (PC-3) cancers can resist shear-induced apoptosis compared to normal cells via Rho-kinase-mediated actin rearrangement (185). Additionally, nuclear lamina proteins lamin A and C, which are key regulators of nuclear stiffness and mechanotransduction, are also essential to breast cancer (MDA-MB-231 and MDA-MB-468) CTC resistance to apoptosis, as their knockdown raises levels of apoptosis to be similar to those of normal breast epithelial (MCF-10A) cells injected into the circulation (186). Altogether, cancer cells can resist high levels of shear and respond to a wide range of shear rates. As the influx of extracellular calcium is not sufficient to trigger most HT-1080 cells to reverse direction, it is possible that another mechanotransduction pathway could independently, or in conjunction with calcium signaling, cause these cells to reverse their migration direction at higher shear rates. Importantly,
higher shear rates are not likely to induce higher intracellular calcium concentrations than those induced by exposure to 10 µM ionomycin, due to the strength of this ionophore and the >10,000-fold increase in typical extracellular calcium concentration compared to typical intracellular calcium concentration (88). Lastly, as the HT-1080 cells migrate significantly faster than all fibroblast lines used here, they have reduced time to transduce mechanical signals prior to exiting the channels, which may allow them to move significantly further before signifying a response.

There is significant evidence that physical force can activate contractility through both RhoA-independent and -dependent pathways. Perhaps the most well studied system for regulation of contractility is in muscle fibers, where increased calcium binds to calmodulin to activate myosin light chain kinase (MLCK), which in turn phosphorylates the regulatory myosin light chain on serine19 and causes the myosin motor to contract against bound actin filaments (94, 95). Influx of extracellular calcium may lead to the elevated levels of P-MLC observed under shear flow, although flow also increased RhoA activity. RhoA can be activated via force transduction through integrins (130), or activated downstream of calcium influx by PKCα activation of p115RhoGEF (153, 154), or through the glyocalyx (293). Once activated, RhoA increases the level of P-MLC by directly inhibiting myosin light chain phosphatase and/or through ROCK (95, 284, 293). Interestingly, fibroblast cells retained some ability to sense and respond to shear flow during inhibition of myosin contractility with blebbistatin, but not during inhibition of ROCK with Y27632, suggesting that other downstream effectors of Rho contribute to cell reversal as well as contractility. Rho also promotes actin stability through ROCK-LIMK-cofilin pathway and induces actin polymerization through the formin mDia1 (284, 302, 306). Rho-mediated cytoskeletal rearrangement may play a role along with cellular contractility in fibroblast reversal of migration direction in response to shear flow. Rho also plays a major role in bleb-based migration modes (68, 306), which were observed in about half of reversing cells. Indeed, studies of intravasation observed the formation of blebs as cells protrude into a vessel, and that bleb formation often accompanies to failure to intravasate (175, 184).
Cell migration is classically believed to be due to coordination of the cytoskeleton by the local activity of RhoGTPases Cdc42 and Rac1 at the cell leading edge and RhoA at the trailing edge \((307, 308)\). However, much of the classical understanding of cell migration comes from studies on 2D surfaces, while migration in confinement and 3D employs significantly different mechanisms \((17, 69, 282, 309)\). Further, accumulating evidence shows that RhoA can also be involved in membrane protrusions at the cell leading edge \((306, 310)\). Fibroblast cells that were exposed to shear flow in 2D showed a nonpolarized increase in RhoA activity in less than an hour. It is not clear whether polarized RhoA activation occurs in fibroblasts exposed to shear stress at the end of microchannels, an experiment that was not performed due to the low throughput of FLIM-FRET imaging. Further evidence of the distribution of RhoA and/or its effectors may lead to a greater understanding of how RhoA mediates reversal. Cdc42 is also activated by increased intracellular calcium \((311, 312)\). RhoA and Cdc42 have several mechanisms for crosstalk \((312)\); during oocyte wound healing, they form mutually exclusive concentric rings of activity \((312, 313)\), which may be mediated by Abr, a protein that localizes with active RhoA and is both a RhoGEF and a Cdc42GAP, thus activating RhoA and inhibiting Cdc42 in the same region. More information on the localization of these two molecules during cell shear exposure and reversal may shed light on their respective roles. Our current hypothesis is that RhoA/myosin and Cdc42 maintain exclusive zones in migrating fibroblasts, and that shear activation of RhoA disrupts established Cdc42 zones at the cell leading edge, often leading to Cdc42 redistribution to the former trailing edge, where its activity causes a reversal of migration direction.

Cancer cell adaptation to and migration against shear stress likely plays a vital role during metastasis. Fibroblast sensation of shear flow may contribute to vascular wound healing, both by limiting fibroblast proximity to the wound prior to clotting, and by stimulating differentiation to myofibroblasts, whose migration is halted by shear flow and whose heightened contractility contributes to wound closure \((162, 293, 298)\). Shear aversion may also be overcome by collective
cell migration, where clusters of cells migrating together prevent leader cells exposed to shear from reversing migration direction. In fact, although fibroblasts are absent from the bloodstream in healthy patients, cancer-associated fibroblasts have been identified circulating in the bloodstream of the majority of prostate and breast cancer patients with metastatic disease (263, 264), often in clusters with tumor cells. This suggests both that fibroblasts can survive for some time in the circulatory system, and that heterotypic collective migration of cancer and fibroblast cells may contribute to intravasation, as described in (253, 255, 314-316).

This study could be improved by implementation of higher shear rates. We expect a dose-dependent increase in the proportion of fibroblasts that reverse their migration direction upon contacting shear flow and are interested to see if cancer cells respond to higher shear stresses. Additionally, the seeding channel is also exposed to fluid shear during experiments, although not until cells have migrated into the microchannels for ~2 hours, thus the analyzed cells should not be influenced by this experimental design. The ionomycin gradient will also later be verified with FITC-dextran imaging to confirm that the device creates stable gradients, as observed in (64). Further work should also be performed to clarify the role of Cdc42 in the reversal process. Assessment of Cdc42 KD cells in the ionomycin gradient experiment will show if Cdc42 is acting downstream of calcium. FLIM-FRET experiments with a Cdc42 sensor could be performed to show its activation in response to shear flow, and the activity’s response to inhibition of potentially upstream regulators (such as calcium channels and RhoA). Overexpression of a kinase dead TRPM7 would clarify the role of the kinase activity versus calcium signaling alone (317). TRPM7 has been demonstrated to regulate contractility in mouse neuroblastoma cells (275). Interestingly, TRPM7 can increase contractility by allowing entry of extracellular calcium or decrease contractility via direct phosphorylation of the myosin IIA heavy chain by the TRPM7 kinase domain (275, 318). Physiological rates of shear flow induce TRPM7 translocation to the nuclear membrane and calcium influx independently of the kinase domain (319). TRPM7 has been implicated in breast cancer migration and metastasis (320, 321) by inhibition of myosin
contractility. This is in line with our previous results indicating that inhibition of myosin contractility in MDA-MB-231 cells increases their migration speed in confinement (15, 18). The activation of myosin contractility observed in this study may be due to a cell-line dependent context for TRPM7, or the convergence of multiple signaling pathways. Assessment of P-MLC levels during inhibition of TRPM7 and/or RhoA would be useful to determine what the upstream regulator of this phosphorylation is. As TRPM7 mediates fibroblast sensation of shear flow, its overexpression in HT-1080 would also be interesting, to see if it sensitizes these cells to shear stress. As cellular wound healing and immune response often involve chemotaxis (130), future work should consider if aversion to shear stress is overcome by chemotaxis stimulus.

Herein we have explored the intersection of physiologically-relevant confined migration in longitudinal tracks and shear stress, such as may occur during cellular intravasation. Fibroblast mechanotransduction of low levels of shear stress activated contractility through a calcium-dependent mechanism to trigger a reversal of cell migration direction. Interestingly, HT-1080 fibrosarcoma cells are insensitive to this level of shear stress and their migration direction is not regulated by the same calcium signaling. This work contributes to our understanding of cell migration that may be relevant to tissue maintenance, wound healing, and cancer metastasis.
Chapter 5

A microfluidic assay for the quantification of the metastatic propensity of breast-cancer specimens

5.1 Introduction

Cancer metastasis is responsible for the vast majority of cancer-related deaths (1). Localized breast cancer has a 99% five-year relative survival rate, which drops to 85% in patients where the disease has spread regionally, and to 27% in patients with distant metastasis (1, 196). In 2018, approximately 266,000 women will be diagnosed with breast cancer in the United States (196). Current estimates reveal that 20-30% of breast cancer patients with early stage disease will eventually experience metastatic recurrence. Exposure of patients at low risk of developing metastasis to aggressive treatments, such as radiotherapy, may compromise the patients’ ability to tolerate further treatment that may be necessary to combat de novo cancer in the future (322). It is estimated that 13,000 women, corresponding to 5% of new diagnoses, will develop de novo metastatic breast cancer in 2018 (196). Thus, it is critical to identify which patients are at risk of developing metastatic disease in order to provide them with effective treatment while also minimizing the overtreatment of patients who are not at risk with potentially harmful and costly therapies.

Current technologies for the prediction or early detection of breast cancer metastasis are limited to gene expression profiling (28) and the quantification of circulating tumor cells (CTCs) (29) or of DNA shed by cancer cells (ctDNA) (27) in the patient’s bloodstream. Gene expression
profiles, such as Oncotype DX, measure the expression levels of a subset of genes and use this pattern to predict prognosis, and in some cases, likelihood of responding to treatment. However, it is unlikely that one panel will be effective for all patients because breast cancer progression can be caused by mutations in different pathways, at different levels within the same pathway, or even at different loci in the same gene (27). Due to the high cost of these tests (>3,000), questions remain about the cost-effectiveness of their use in the clinic (234). Detection of CTCs using the FDA-approved CellSearch system has prognostic value for predicting disease free survival and overall survival. However, current implementation of the technology still suffers from low sensitivity and specificity for predicting patient outcomes (241). Detection of ctDNA is typically performed by sequencing primary tumor DNA and then developing polymerase chain reaction (PCR) probes for unique characteristics of the tumor genome (e.g., somatic mutations or chromosomal rearrangement). This approach has been applied in early studies to predict the recurrence or metastasis of breast cancer with high specificity but has been limited by its sensitivity to detect ctDNA (31-80%) (242). Detection of CTCs or ctDNA is minimally invasive and has the potential to monitor a patient’s response to treatment after it is administered, but neither approach can predict whether a patient will respond to specific therapeutic regimens (27). Improved sensitivity, lead time in prediction of metastasis before its clinical detection, and ability to screen therapeutic regimens for patient-specific effectiveness will improve patient outcomes.

Cells within a tumor are heterogeneous; it is believed that only a tiny fraction of cells within a primary tumor is capable of forming metastases (223). The identification and isolation of these metastasis-initiating cells would enable the prediction of a patient’s risk of developing metastasis and the design of optimal, personalized therapeutic treatments. Metastatic cells are bestowed with a repertoire of distinct abilities that enable them to separate from the primary tumor, locally invade the surrounding stroma, intravasate and survive in the circulatory system, roll and arrest on a vessel wall, extravasate, locally invade and form a pro-metastatic niche, and
finally proliferate to colonize a distant organ. Clearly, cell migration and survival/proliferation represent integral components of the metastatic cascade \((1, 8)\). In vivo, metastasizing cells migrate through pores in the stromal extracellular matrix (ECM), longitudinal tracks created by ECM degradation (by cancer-associated stromal cells or cancer cells themselves) or formed between the basement membrane and tissue, and along blood vessels or ECM fibrils \((8)\). The cross-sectional area of these in vivo pores/tracks ranges \((45)\) from 10-300\(\mu\)m\(^2\). Following migration in confinement and arrival at a distant site, metastatic cells must proliferate to generate a secondary colony. We herein developed a Microfluidic Invasion Network Device (MIND), which enables us to concurrently measure the relative abundance of migratory cells and their proliferation state in an effort to identify cells capable of forming metastatic lesions. MIND consists of two parallel seeding and collection channels connected by Y-shaped microchannels that mimic aspects of the complexity and variety of cross-sectional areas observed in vivo \((Fig. 5-1A)\). The Y-shaped channels have a relatively large feeder channel (with width, \(W\), of 20\(\mu\)m and height, \(H\), of 10\(\mu\)m) to maximize the number of cells that enter the microchannels for study. Cells that reach the far end of the feeder channel encounter a bifurcation region and must choose between two narrower branch channels \((W\) of 10\(\mu\)m or 3\(\mu\)m with a fixed \(H\) of 10\(\mu\)m). The MIND assay uses time-lapse microscopy to perform high-throughput screening of numerous cells in less than 24h. The assay requires only 50,000 cells to accurately and reproducibly detect the rare subpopulation of metastasis-initiating cells, which may constitute less than 0.1% of the tumor population \((\leq 50\) out of 50,000 cells) \((2)\). Furthermore, migratory cells can be physically isolated from a heterogeneous tumor specimen for molecular and genetic characterization. Altogether, MIND possesses the advantages of requiring a small sample, delivering rapid results, screening the effect of therapeutics on highly-motile metastasis-initiating cells, and physically isolating these cells for further characterization. We herein demonstrate the potential of MIND for diagnosis and precision care in breast cancer.
5.2 Materials and Methods

5.2.1 Microfluidic Invasion Network Device (MIND) assay. Microfluidic devices were fabricated and seeded with 50,000 cells as described previously (64, 323, 324). Briefly, microchannels were patterned in polydimethylsiloxane (PDMS, Sylgard 184) by replica molding using master molds created on silicon wafers (Wafer World, Inc.) by photolithography. Patterned PDMS was cleaned, activated by oxygen plasma in a Harrick PDC-32G plasma cleaner (Harrick Plasma), and bonded to glass slides (Electron Microscopy Sciences). Assembled microfluidic devices were immediately coated with 20µg/mL collagen type-I (BD Biosciences). Migration experiments were performed in DMEM plus 10% (v/v) fetal bovine serum (FBS, Life Technologies/Gibco) and 1% (v/v) penicillin/streptomycin (P/S, Gibco). Time-lapse images were recorded in 20min intervals for 24h using a Digital Sight Q1Mc camera mounted on an inverted Eclipse Ti Microscope (Nikon) equipped with a 10x/0.30 numerical aperture lens.

5.2.2 Transwell-migration assay. Cell migration was monitored using the xCELLigence RTCA DP device (Acea Biosciences, Inc.) using the manufacturer’s protocol. Briefly, 40,000 cells per well were added to the upper chamber of a CIM-Plate 16. These plates have chambers that are similar to a Boyden chamber; they consist of an upper chamber where the cells are seeded in serum-free DMEM with 1% P/S, a microporous polyethylene terephthalate (PET) membrane (average pore diameter of 8µm), electrodes directly below this membrane to detect cell migration, and a lower chamber that was filled with DMEM containing 10% FBS as a chemoattractant and 1% P/S. Migration progress was recorded in real-time as cell index (a change in the electrical impedance between the electrodes caused by migrating cells). Measurements were taken every 15 minutes for 48 hours in an incubator maintained at 37°C and 5% CO₂. Each cell type was run in triplicate for each experiment.
5.2.3 Cell lines used in this study, and viability measurements. Immortalized human mammary epithelial cells (HMLE) were transduced with a retrovirus carrying pMSCV-Luciferase PGK-hygro expression vector (Addgene 18782, used for all luciferase-expressing cell lines). Single HMLE clones were isolated and the clones with the brightest bioluminescent signal were selected. To ensure that results were not due to clonal variance, two of the brightest clones, HMLE Luc 26 and 8, were selected at random and used in this project. These cells were grown in Mammary Epithelial Cell Growth Medium (Lonza). 184B5 cells were grown in Mammary Epithelial Cell Growth Medium plus 1 ng/mL cholera toxin (Sigma-Aldrich). 184A1 cells were grown in Mammary Epithelial Cell Growth Medium plus 1 ng/mL cholera toxin and 5 µg/mL transferrin (Sigma-Aldrich). MCF-10A cells and variants were created and cultured as described in (325). MCF-12F cells were a generous gift from Dr. Denis Wirtz (Johns Hopkins University) and were grown in the same media as the MCF-10A cells. HCC1428 and ZR75-1 cells were grown in RPMI 1640 plus 10% and 1% P/S. MDA-MB-468, MCF7, T47D, MDA-MB-436, Hs578t, BT-549, and MDA-MB-231 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies/Gibco) plus 10% FBS and 1% P/S. SkBr3 cells were grown in McCoy’s 5A (Modified) Medium (Gibco) plus 10% FBS and 1% P/S. BT20 cells were grown in Minimum Essential Medium Eagle (Sigma-Aldrich) plus 10% FBS and 1% P/S. SUM159 cells were a generous gift from Dr. Dipali Sharma (Johns Hopkins University); they and SUM149 cells were grown in Ham’s F-12 Medium (Corning Cellgro) plus 5% FBS, 1% P/S, 1µg/mL hydrocortisone (Sigma-Aldrich), and 5µg/mL insulin (Sigma-Aldrich). MCF7 targeted WT and MCF7 Her2 were a generous gift from Dr. Ben Ho Park (Johns Hopkins University) and were cultured as described in (136). The MCF7 cell genome contains two copies of an activating E545K mutation in the PIK3CA gene, and one wild-type copy of this gene. The MCF7 targeted WT cells have had these alleles corrected to wild-type (136). MCF7 Her2 cells have one wild-type allele of HER2 and one activating mutant V777L allele (136). MCF7-Luciferase cells were transduced with lentivirus for luciferase expression and have been shown to be non-metastatic. These cells were
grown in DMEM plus 10% FBS, 1% P/S, and 5µg/mL puromycin (Gibco). MDA-MB-231 Tumor, LungMet, and CTC variants were grown in DMEM plus 10% FBS, 1% P/S, and 125µg/mL hygromycin (ThermoFisher). To produce these cell lines, MDA-MB-231 cells were transduced with lentivirus for luciferase expression and injected into the mammary fat pads of mice. Cells from the primary tumor were dissociated and human cells were selected with hygromycin (MDA-MB-231 Tumor). Single cells were dissociated from secondary tumors formed in the lung and human cells were selected with hygromycin (MDA-MB-231 LungMet). Blood samples were taken and a CTC cell was isolated (MDA-MB-231 CTC). Cells were maintained in a humidified incubator at 37°C, 95% air/5% CO₂. Cells were routinely checked for mycoplasma contamination via PCR using the primers: F-(5’-GGGAGCAACAGGATTAGATACCCT) and R-(5’-TGCACCATCTGTCACTCTGTAAACCTC). Unless otherwise specified, cell lines were purchased from the American Type Culture Collection (ATCC). Cell viability measurements were performed as previously described (325).

5.2.4 Classification of cell lines. Cell lines were classified as having low or high metastatic potential based on reports of their ability to consistently metastasize in mouse models (spontaneously or following injection into the circulatory system) (326-329).

5.2.5 Identification of threshold. The threshold percentage of migratory cells and time were systematically varied over a range of 1-20% and 1-24h. For each combination of values, the percentage of migratory cells from each cell line was compared to the threshold percentage to see if MIND predicts that the cell line would have low metastatic potential (<threshold) or high metastatic potential (≥threshold). MIND’s predictions were compared to our assessment of the cell lines (Table 5-2), and used to classify each prediction as true positive, true negative, false
positive, or false negative (where true denotes a match between MIND’s prediction and cell line classification, and positive/negative denotes high/low metastatic potential, respectively). Using these classifications, the sensitivity (percentage true positive predictions out of number of cell lines with high metastatic potential), specificity (percentage true negative predictions out of number of cell lines with low metastatic potential), and accuracy (percentage of true predictions out of the total number of cell lines tested) of MIND’s predictions were calculated for each combination of threshold and time.

5.2.6 Immunostaining. Cells were fixed, permeabilized, blocked against nonspecific adhesion, immunostained for target proteins, and then imaged on an inverted Eclipse Ti epifluorescence microscope (Nikon). Primary antibodies were administered at manufacturer recommended concentration: anti-CD45 (clone HI30, Becton Dickinson), anti-human mitochondria (clone 113-1, MilliporeSigma), anti-Ki67 (clone 8D5, Cell Signaling Technology), and anti-phospho-histone H2A.X (Ser139, clone 20E3, Cell Signaling Technology). The secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen) and Alexa Fluor 568 goat anti-rabbit IgG (H+L) (Invitrogen).

5.2.7 Logistic regression. Logistic regression was used to calculate the probability of a cell line having high metastatic potential based on the predictors \(X_i\) percentage migratory cells and percentage Ki-67-positive cells (eq.1). Logistic regression coefficients \(b_i\) were trained in MATLAB using the glmfit function for the panel of 25 breast epithelial and breast cancer cell lines (Table 5-1). Probability values were calculated in MATLAB using the glmval function.

\[
p = \frac{e^{(b_0 + b_1X_1 + b_2X_2)}}{1 + e^{(b_0 + b_1X_1 + b_2X_2)}} \times 100 \tag{1}
\]
Table 5-1. Logistic regression coefficients developed from panel of breast epithelial and breast cancer cell lines.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Logistic regression coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant term (B0)</td>
<td>-2.846</td>
</tr>
<tr>
<td>Migratory cells (%)</td>
<td>37.82</td>
</tr>
<tr>
<td>KI-67 positive cells (%)</td>
<td>41.72</td>
</tr>
</tbody>
</table>

5.2.8 CellTracker labelling. MDA-MB-231 and MCF7 cells were labelled with either Red CMTPX dye or Green CMFDA dye (Invitrogen) according to the manufacturer’s protocol. The color assigned to each of the cell types was randomized for each experiment. Briefly, the media was aspirated from 60-80% confluent cells in a T25. The cells were washed with PBS, and then incubated with 2mL of DMEM containing 1µM CellTracker dye at 37°C for 30min. After incubation, cells were passaged and used for the experiment.

5.2.9 Isolation of migratory cells. 50,000 MDA-MB-231 cells were seeded in MIND and incubated for 24h. Migratory cells that exited the branch channels and entered the collection channel were washed with PBS and then detached from the device with 0.05% trypsin-EDTA (or TrypLE Express for RNA isolation experiments). Microbore tubing was attached to a collection inlet, and connected to a sterile 0.2 µm filter (Corning) and a 10mL syringe (Beckton Dickinson) containing DMEM plus 10% FBS and 1% P/S. Observing under a microscope, media was pushed from the syringe through the collection channel to force the migratory cells into the collection outlet. These cells were collected with a micropipette and transferred to a 15mL Falcon tube contained 5mL of media. Each microfluidic device typically yielded 50-100 migratory cells. Cells from multiple microfluidic devices were pooled to generate greater numbers of migratory cells. To collect a control population, cells from the seeding channel were dissociated and collected in a similar manner to the migratory cells. Since 50,000 cells were seeded in the MIND assay,
thousands of cells adhere to the device, of which only hundreds are positioned close enough to the feeder channels to migrate, and of which an even smaller fraction are migratory, collection of cells from the seeding channel yields essentially the unsorted population, which contains both non-migratory (~80%) and migratory (~20%) cells. Thus, for this experiment, comparison was made between the migratory cells and the unsorted population.

5.2.10 Subcutaneous injection and bioluminescent imaging. All animal studies were performed following Institutional Animal Care and Use Committee procedures and guidelines at University of Maryland, Baltimore under an approved protocol. Eight-to-twelve-week-old female NOD SCID mice weighing 19-25g were obtained from Charles River (Fredrick, MD) and fed food and water ad libitum. Animals were randomly assigned to groups. For subcutaneous injections, equal numbers (500) of migratory or unsorted luciferase-tagged MDA-MB-231 cells were suspended in 100µL PBS and mixed with an equal volume of Matrigel (Corning). Cell number was quantified via hemocytometer and confirmed by bioluminescent imaging.

Bioluminescence was only detected in viable cells expressing the firefly luciferase gene, indicative of an active metabolism. At the indicated time points following injection, mice were injected intraperitoneally with Luciferin (150 mg/kg, Perkin Elmer) and returned to their cages for 5 min to allow for biodistribution. Mice were anesthetized with 2% isoflurane gas and imaged at 5-min intervals for the maximum photon emission. Total 60 photon flux (photons/s) was calculated and corrected for tissue depth by spectral imaging using Living Image 3.0 software (IVIS). To quantify the bioluminescent signal from each organ, total bioluminescent signal was measured using regions of interest of equal areas for comparison between migratory and unsorted cell samples, and the background signal was subtracted. To avoid false positive detection of bioluminescent signal, the background subtracted value was normalized to the background reading, and only readings that were ≥ 50x the background reading were considered to be positive.
for metastasis. In order to control for differences in tumor size, bioluminescent values were normalized to the volume of the primary tumor at the time of necropsy.

5.2.11 qPCR. qPCR for hLINE was conducted as described previously (189). Dilutions of human DNA isolated from MDA-MB-231 cells were included in each plate for comparison. Primers used for verification of DEG were:

- *FGF5* F-(5′-CTCTTCCCCCTCTCCCTCTTCT) and R-(5′-GGCTGATTCTGGGCTCTGTA),
- *CCND1* F-(5′-GGATGCTGGAGGTCTGCGA) and R-(5′-AGAGGCCACGAACATGCAAG),
- *TAB2* F-(5′-GACCTGCCTGGAAAGAAGT) and R-(5′-CTCTTCTGTCCAAGCATTITCTTG),
- *AKT2* F-(5′-GCTCCACAACGCTGGGTAAT) and R-(5′-GGCCCTCTCGGTCTTCATCAG),
- *KRAS* F-(5′-CATTGGTGAGGAGATCCCGA) and R-(5′-AGGCATCATCAACACCCAGAT),
- and *FOS* F-(5′-TAGTTAGTAGCATGGTGAGCCAGG) and R-(5′-ACCACCTCAACAATGCATGA).

5.2.12 Immunohistochemistry. Animals with primary tumor formation that exceeded the designated endpoint, including saturation exceeding 1000-fold over the initial bioluminescence signal, were sacrificed. Tissue samples were removed, fixed in formalin for 24 h, embedded in paraffin wax, and serially sectioned (4-μm thick). All immunohistochemistry and H&E staining was performed by Mass Histology Services (Worcester, MA).

5.2.13 Cell tracking. Cells were tracked manually every 20 min using ImageJ software (National Institutes of Health) using the Manual Tracking plugin (324). In select experiments, polygonal regions of interests (ROIs) were manually drawn around the cell periphery in 40 min intervals using ImageJ and saved to the ROIs Manager. The Measure ROI function was then used to
calculate the cell area, aspect ratio and solidity (324). Values of each metric were averaged over the time the cell spent in the feeder channel.

5.2.14 RNA sequencing and analysis. RNA was isolated from samples of 1000 migratory or unsorted cells in triplicate using the Nucleospin RNA XS kit (Macherey-Nagel). cDNA libraries were amplified using the SMART-seq ultra low input RNA kit (Takara) and then tagmented and barcoded by indexing primers using Nextera XT DNA library prep kit (Illumina). Samples were pooled and paired-end sequenced on an Illumina NextSeq 500 using the NextSeq 500/550 Mid Output v2 kit with 150 cycles and an output of up to 130 million reads. After quality control of raw data using Illumina pipeline, RNA-seq reads were mapped to hg38 reference genome using HISAT2 (330) aligner. Htseq-count command from the HTSeq framework (331) was used to quantify read counts per gene from aligned reads using human ENSEMBL 86 (GRCh38.p7) gene models. The Bioconductor/R package DESeq2 (332) was used for normalization and differential gene expression analysis. Pathway analysis and gene ontology clustering were performed using the Database for Annotation, Visualization, and Integrated Discover (DAVID) (333, 334).

5.2.15 DNA damage quantification. Cells were imaged on a Nikon A1 confocal microscope using a Plan Apo 60x objective (NA=1.4). ImageJ was used to convert images to 8-bit, set a binary threshold, and quantify the number and area of foci.

5.2.16 Tail vein injection. Eight-to-twelve-week-old female athymic nude-Foxn1nu mice weighing 19-25g were obtained and fed as described above. MCF-10A, 10A-KRAS(G12V), PTEN, and PTEN−/−KRAS (G12V) cells (1×10⁶ cells/100µL) were injected intravenously in
coorts of 5 animals. Animals were monitored by bioluminescence signal and carried out through 20 weeks or until recurrent growth (observed only in mice injected with PTEN−/−KRAS cells) required the mouse to be sacrificed. After 20 weeks, mice injected with MCF-10A, PTEN−/−, or KRAS(G12V) cells still survived and were monitored for recurrence and clinical distress by gross examination.

5.2.17 Patient-Derived Xenograft specimens. Cryogenically preserved tumor specimens were implanted into the cleared mammary fat pads of NOD-SCID mice. Both samples formed tumors, which were excised, dissociated to single cells as described in (335), and allowed to recover on collagen-I coated tissue culture dishes in DMEM plus 10% FBS and 1% P/S for 48h. Cells were then seeded in MIND and monitored via time-lapse microscopy for 24h. After imaging, cells were fixed and immunostained for human mitochondria and other markers. To exclude the possibility of the dissociated cells being human leukocytes preserved during resection from the patient, select samples were immunostained for CD45, and were negative in all cases (data not shown). To assess Ki-67-positive cells, previously stained samples were quenched with 10mg/mL sodium borohydride (Sigma-Aldrich) in PBS for 15 min. Samples were then rinsed thoroughly with PBS, blocked and immunostained for Ki-67 and human mitochondria (AB3598, MilliporeSigma).

5.2.18 Western blotting. Western blots were performed as described in (191). Primary antibodies were administered at the manufacturer recommended concentration: anti-actin (clone Ab-5, Becton Dickinson), anti-Akt (clone C67E7, Cell Signaling Technologies), anti-pAkt (Ser473) (clone D9E, Cell Signaling Technologies), anti-Erk (clone L34F12, Cell Signaling Technologies), anti-pErk (clone D13.14.4E, Cell Signaling Technologies). Secondary antibodies: anti-mouse IgG
HRP-linked antibody (Cell Signaling Technologies), anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies).

5.2.19 Statistical methods. Data means±SEM were calculated and plotted using GraphPad Prism 7 (GraphPad Software). The D’Agostino-Pearson omnibus normality test was used to determine if data are normally distribution. Data sets with gaussian distributions were compared using an unpaired student’s t test (two-tailed) or ANOVA followed by Tukey’s multiple comparisons test. Data sets with nongaussian distributions were compared using an unpaired Mann-Whitney test (two-tailed).

5.2.20 Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request. RNA-seq data is available at NCBI GEO (GSE128313).
Figure 5-1: Use of MIND for prediction of metastatic potential of breast epithelial and breast cancer cell lines with high accuracy, sensitivity and specificity. (A) Schematic of Microfluidic Invasion Network Device (MIND). Inset: Migratory (closed triangle) and non-migratory (open triangle) MDA-MB-231 cells. (B) Percentage of migratory cells from normal-
like breast epithelial and breast cancer cell lines at 13h. Each data point represents the percentage from 1 experiment. Column and error bars represent mean±SEM of n≥3 independent experiments. Dotted line designates 7% of migratory cells. (C) Sensitivity, specificity and accuracy (%) of prediction of metastatic potential of established cell lines based on the mean percentage of migratory cells. Metrics are calculated as a function of experiment duration (0-24h) and threshold (0-20%) above which a cell line is predicted to have high metastatic potential. White circles represent the points corresponding to maximum accuracy (96%) for the motility index. (D) ROC of metastatic potential predictions based only on migration index. (E) Percentage of Ki-67-positive cells observed in MIND. Each data point represents the percentage from 1 experiment. Column and error bars represent mean±SEM of n≥3 independent experiments. Dotted line designates 55% of Ki-67-positive cells. (F) ROC based only on proliferation index. (G) Probability (%) of each cell line possessing high metastatic potential calculated using logistic regression and the percentages of migratory and Ki-67-positive cells. Baseline value (shown in white) for each heat map is set to the threshold value for each predictor (7% migratory cells and 55% Ki-67-positive cells). (H) ROC of MIND using the combined migration and proliferation indices.
5.3 Results

5.3.1 Use of MIND for prediction of metastatic potential of breast epithelial and breast cancer cell lines with high accuracy, sensitivity and specificity. To determine MIND’s ability to distinguish between breast cancer cell lines with high versus low metastatic propensity or normal-like breast epithelial cells, we first examined the migratory potential of a large panel of established cell lines (Table 5-2). Specifically, we analyzed cells that entered the feeder channel and classified them into two categories: as non-migratory if their locomotion was limited only to the feeder channel, or as migratory if they reached the bifurcation region and entered one of the branch channels (Fig. 5-1A). Highly metastatic breast cancer cell lines contain a larger fraction of migratory cells than that of breast cancer cells with low metastatic potential or normal-like breast epithelial cells (Fig. 5-1B, Table 5-2). These data indicate that the relative abundance of migratory cells in a heterogeneous population correlates with its metastatic potential.
Table 5-2: Panel of established normal-like breast epithelial cells and breast cancer cell lines with either low or high metastatic potential used to validate MIND. Estrogen receptor (ER), progesterone receptor (PR), and Her2 status are indicated. Mean percentage of migratory and Ki-67 cells from $n\geq3$ independent experiments. Probability ($P$) of having high metastatic potential calculated with logistic regression formula using migration and proliferation indices as predictors.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>Site of origin</th>
<th>ER</th>
<th>PR</th>
<th>Her2</th>
<th>% mig</th>
<th>% Ki67</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HMLE Luc 26</td>
<td>None</td>
<td>Mammary gland/epithelium</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>0.5</td>
<td>48.4</td>
<td>0.00</td>
</tr>
<tr>
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<td>Neg</td>
<td>Neg</td>
<td>1.6</td>
<td>33.1</td>
<td>0.00</td>
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<tr>
<td>184B5</td>
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<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1.5</td>
<td>2.0</td>
<td>0.00</td>
</tr>
<tr>
<td>184A1</td>
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<td>Mammary gland/epithelium</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>5.7</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
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<td>Fibrocystic disease</td>
<td>Mammary gland</td>
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<td>Neg</td>
<td>Neg</td>
<td>1.1</td>
<td>32.9</td>
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</tr>
<tr>
<td>MCF-12F</td>
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<td>Mammary gland</td>
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<td>Neg</td>
<td>Neg</td>
<td>3.3</td>
<td>32.6</td>
<td>0.00</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCC1428</td>
<td>Adenocarcinoma</td>
<td>Metastatic site: pleural effusion</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>0.0</td>
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<tr>
<td>ZR75-1</td>
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<td>Metastatic site: ascites</td>
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<td>Neg</td>
<td>0.0</td>
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</tr>
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<td>Metastatic site: pleural effusion</td>
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<td>0.0</td>
<td>62.0</td>
<td>0.00</td>
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<tr>
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<td>Pos</td>
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<tr>
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<td>Adenocarcinoma</td>
<td>Metastatic site: pleural effusion</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>1.2</td>
<td>59.6</td>
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<td>Metastatic site: pleural effusion</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>2.9</td>
<td>47.5</td>
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<tr>
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<td>Adenocarcinoma</td>
<td>Metastatic site: pleural effusion</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>5.4</td>
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<td>Pos</td>
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<td>6.5</td>
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<td>T47D</td>
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<td>Metastatic site: pleural effusion</td>
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<td>Pos</td>
<td>Neg</td>
<td>4.2</td>
<td>21.3</td>
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<td>Primary tumor</td>
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<td>Neg</td>
<td>Neg</td>
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<td>Xenograft - mammary fat pad</td>
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<td>15.4</td>
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<td>Xenograft - CTC</td>
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<td>Neg</td>
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<tr>
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In light of these observations, we sought to determine a threshold percentage of migratory cells that separates cell populations with low versus high metastatic potential. Since the percentage of migratory cells is a function of time (Fig. 5-2A), we optimized both the threshold percentage and experiment duration to maximize the sensitivity, specificity and accuracy of MIND. MIND has 100% sensitivity to detect cell populations with high metastatic potential at low threshold percentages and long durations (Fig. 5-1C). At early time points, sensitivity is reduced because migratory cells have not had sufficient time to reach and enter the branch channels. Conversely, 100% specificity is achieved at higher threshold percentages and shorter times (Fig. 5-1C). At later time points, cells from cell lines with low metastatic potential that struggle to reach the bifurcation and/or squeeze into the narrower branch channels have increased chance to succeed and be classified as migratory. The total accuracy of MIND is maximized in the middle of these parameter ranges (Fig. 5-1C). With a narrow range of threshold percentage (7-9%) and experiment duration (12-14h), we obtained optimal accuracy (96%) with high sensitivity (89%) and specificity (100%) (Fig. 5-1C, Fig. 5-2B). This corresponds to a positive predictive value of 100% and a negative predictive value of 96% (Fig. 5-2C-D). This analysis produced one false-negative result, occurring for the SUM149 cell line. For 13h, the MIND assay’s receiver operating characteristic (ROC) is plotted in Fig. 5-1D and has 94% area under the curve (AUC), demonstrating the ability to achieve high sensitivity without compromising specificity.
Figure 5-2: Use of MIND for prediction of metastatic potential of breast epithelial and breast cancer cell lines. (A) Percentage of migratory cells as a function of time in representative cell lines with high metastatic potential (MDA-MB-231), low metastatic potential (MCF-7), and normal-like breast epithelial cells (MCF-10A). Data represent mean±SEM from n=3 independent experiments. *, p<0.05 compared to MCF-10A and MCF-7, as calculated by two-way ANOVA followed by Tukey’s multiple comparisons test. (B) Combinations of threshold percentage of
migratory cells and experiment duration that result in optimal accuracy for MIND based only on migration index. (C) Positive predictive value (PPV, sum of true positive predictions divided by sum of total assay positive predictions) and (D) negative predictive value (NPV, sum of true negative predictions divided by sum of total assay negative predictions) of predictions based on migration index as a function of experiment duration and threshold percentage. At early time points, the assay makes no positive predictions and PPV is undefined (white region). White circles represent the values corresponding to optimal accuracy (96%). (E) Cell index (proxy for number of cells that migrated at 24 h) of breast epithelial and breast cancer cell lines measured in a transwell-migration assay using the xCELLigence RTCA DP instrument with CIM-plate 16 chambers. Each data point represents the cell index from 1 experiment. Column and error bars represent mean±SD of n≥3 independent experiments. Dotted lines designate cell index of 2 and 3. (F) Combinations of threshold cell index and experiment duration that result in maximal accuracy of prediction of metastatic potential of established cell lines based on the mean cell index from the transwell-migration assay. (G) Sensitivity, specificity and accuracy (%) of prediction of metastatic potential of established cell lines based on the mean cell index from the transwell-migration assay. Metrics are calculated as a function of experiment duration (0-48h) and threshold (0-4) above which a cell line is predicted to have high metastatic potential. (H) ROC of metastatic potential predictions based on cell index from the transwell-migration assay. (I) MDA-MB-231 cells in MIND after immunostaining for Ki-67 and the nucleus (Hoechst 33342). Scale bars, 50µm. (J) Prediction sensitivity, specificity and accuracy based on proliferation index only at different threshold values of percent Ki-67-positive cells. (K) Percentage of Ki-67-positive cells or (L) migratory cells at 13h observed in MIND filled with DMEM+10%FBS+1%P/S (DMEM) versus each cell line’s native media (listed in Materials and Methods). Columns and error bars represent mean±SD of n=3 independent experiments.
To compare the performance of the MIND assay to that of conventional migration assays, we also evaluated this panel of cell lines in a transwell-migration assay using the xCELLigence RTCA DP instrument with CIM-plate 16 chambers. These plates have chambers that are very similar to Boyden chambers with an average pore diameter of 8 µm and are equipped with electrodes that measure cell migration via changes in electrical impedance, thereby allowing for measurements in real-time. The 8 µm diameter pores were selected for two reasons: 1) these pores have a cross sectional area of ~50 µm², which is within the range of 30-100 µm² of MIND’s branch channels; 2) cell passage through 3 µm, but not 8 µm, pores causes significant DNA damage, which portends genome variation (336, 337), thereby potentially altering the molecular signature and phenotype of cells. The readout of cell migration from this instrument, cell index, was not strikingly different between breast cancer cell lines with low versus high metastatic potential (Fig. 5-2E). Analysis of the experiment duration and cell index threshold value to optimize the sensitivity, specificity and accuracy of this assay is shown in Fig. 5-2F-G. The transwell migration’s ability to predict metastatic potential revealed significantly reduced accuracy of 72% (Fig. 5-2F-G) as compared to 96% for migration alone in MIND. This reduced accuracy corresponded to similar specificity (94-100% for transwell compared to 100% for MIND), but markedly reduced sensitivity (22-33% for transwell compared to 89% for MIND) (Fig. 5-2F-G). The tradeoff between optimizing sensitivity versus specificity is greater for the transwell-migration assay, corresponding to the reduced AUC (70%) of ROC (Fig. 5-2H) compared to 94% for MIND. Altogether, our data reveal that the MIND assay is better suited to identify cell lines with low versus high metastatic potential.

To further improve the predictive power of the MIND assay, we examined the benefit of incorporating an additional index distinct from migration into our analysis. Ki-67 is a protein found in the nucleus of actively proliferating cells that is already used clinically to evaluate breast cancer patient prognosis (338, 339). Because proliferation is necessary for the establishment of
new metastatic colonies, we examined the percentage of Ki-67-positive cells in MIND using immunofluorescence (Fig. 5-2I) and found that cell lines with high metastatic potential tend to have a higher percentage of Ki-67-positive cells (Fig. 5-1E, Table 5-2). The percentage of Ki-67 cells has satisfactory predictive power (Fig. 5-1F), with maximal accuracy of 88% (Fig. 5-2J). Although the majority of cell lines were cultured in DMEM, a few were cultured in other media as recommended by ATCC. However, neither the percentage of Ki-67-positive cells nor of migratory cells were altered by the different media (Fig. 5-2K-L). In view of these findings, we next evaluated the combination of Ki-67 as a proliferation index with the percentage of migratory cells as a motility index. We incorporated both indices as predictors in a logistic regression formula to predict the probability of a cell line having high metastatic potential (Fig. 5-1G). This probability correctly identifies the metastatic potential of every cell line from the panel (Fig. 5-1G), corresponding to a ROC curve with 100% AUC (Fig. 5-1H). Taken together, these results indicate that the MIND assay accurately predicts the metastatic potential of breast epithelial and breast cancer cell lines.

To determine the ability of MIND to identify migratory cells within heterogeneous populations, we mixed at various ratios (1:1, 1:4, and 1:9) aggressive MDA-MB-231 breast cancer cells with non-aggressive MCF7 breast cancer cells pre-labeled with two spectrally distinct fluorophores and seeded a total of 50,000 cells in MIND. The percentage of migratory cells as a function of time was quantified for the mixed population, as well as for the 2 cell lines individually within each device (Fig. 5-3A-C). Even when MDA-MB-231 cells are diluted 10-fold, about 20% of these MDA-MB-231 cells are still migratory. Along these lines, the percentage of MCF7 migratory cells is below the threshold for all different ratios examined in this work.
Figure 5-3: Identification of migratory cells within heterogeneous cell populations. Percentage of migratory cells from mixed populations of MDA-MB-231 and MCF7 cells pre-labeled with two spectrally distinct fluorophores. Fluorescent cell labelling allowed each cell line to be analyzed individually, as well as the mixed population. Data represent mean±SD from n=3 independent experiments. A total of 50,000 cells were seeded in each device, at ratios of (A) 1:1 MDA-MB-231 cells to MCF7 cells (B) 1:4 (C) 1:9. P-values calculated by two-way ANOVA followed by Tukey’s multiple comparisons test. *, p<0.05 for MDA-MB-231 compared to MCF7 at the same time point. $, p<0.05 for MDA-MB-231 and Total compared to MCF7. #, p<0.05 for MDA-MB-231 compared to MCF7 and Total.
5.3.2 Migratory cells have similar tumorigenic but markedly increased metastatic potential in vivo than unsorted breast cancer cells. Since the relative abundance of migratory cells is integral to the prediction of metastatic potential, we hypothesized that these cells have an elevated capacity to form metastases compared to the unsorted cell population. It is noteworthy that isolated migratory cells lose their migration advantage over the heterogeneous unsorted population after cell culture in vitro for 14 days (Fig. 5-4A). To avoid genetic and phenotypic changes caused by in vitro culture on stiff, 2D vessels (340), we performed in vivo assessment of tumor formation and spontaneous metastasis of migratory cells versus unsorted cells directly after isolation using MIND. Isolated migratory or unsorted cells were subcutaneously injected into the fourth mammary fat pad of NOD-SCID gamma mice. Bioluminescent imaging revealed that both cell populations formed tumors that grew at similar rates (Fig. 5-5A, Fig. 5-4B).
Figure 5-4: Migratory cells have enhanced metastatic, but not tumorigenic, potential in vivo compared to unsorted breast cancer cells. (A) Percentage of migratory cells from migratory and unsorted populations cultured for 14 days after isolation from MIND. Data represent mean±SEM from $n=3$ independent experiments. (B) Representative bioluminescent images of tumor growth in mice injected with migratory or unsorted cells. (C) Standard curve for threshold cycle detection of human DNA isolated from MDA-MB-231 cells and detected using qPCR. For each experiment, the average value of three technical replicates was used. Data represent mean±SEM from $n=3$ independent experiments. Line represents best fit, $R^2>0.99$. 
Figure 5-5: Migratory cells have similar tumorigenic but markedly enhanced metastatic potential in vivo than unsorted breast cancer cells. (A) Bioluminescent signal from migratory and unsorted cell xenografts. Percent growth was determined by subtracting background from the peak signal on the appropriate day and normalizing to the initial background subtracted reading.
for the same mouse. Data represent mean±SEM from \( n=8 \) mice per group. (B) Representative bioluminescent images of the lung, liver, axillary lymph node (LN), and bone of mice injected with migratory or unsorted cells. (C) Quantification of bioluminescent signal from lung, liver, LN, and bone. To prevent false-positive readings, each site was considered positive if its peak bioluminescent signal minus background was \( \geq 50x \) background. This fold increase over background was normalized to tumor volume to control for variation between specimens. Each data point represents the signal from 1 mouse. Column and error bars represent mean±SEM from mice with detectable metastases (\( n \) mice annotated in each bar). (D) Amount of human DNA detected by qPCR following isolation from lungs or (E) liver of mice injected with migratory or unsorted cells. Each data point represents the reading from 1 mouse. Data represent mean±SEM from \( n=5 \) mice per group. Lungs/liver from mice not injected with tumor cells served as negative controls. (F) Representative 20x images of immunohistochemistry for human mitochondria and hematoxylin and eosin (H&E) staining of adjacent sections from the lung, liver, and LN of mice injected with migratory or unsorted cells. Insets show the surrounding area at 4x, and indicate the area displayed at 20x. *, \( p<0.05 \) as assessed by two-tailed Mann-Whitney test. NS, \( p\geq0.05 \) as assessed by two-tailed Mann-Whitney test.
Bioluminescent imaging analysis reveals that 4/8 mice injected with migratory cells developed metastases in the bone after 8 weeks, whereas no mice injected with cells from the unsorted population metastasized to this tissue (Fig. 5-5B-C). Moreover, 7/8 mice injected with migratory cells formed metastases in the lung and liver, as opposed to 6/8 or 5/8 mice injected with the unsorted cell population, respectively (Fig. 5-5B-C). Intriguingly, quantitative image analysis reveals an 8-fold increase in the metastatic burden of the lung and liver of mice injected with migratory cells relative to those injected with the unsorted cell population. To validate the differences in metastatic burden observed in the lung and liver, DNA was isolated from samples of these tissues and the amount of human DNA was quantified via qPCR using primers specific for human Long Interspersed Nuclear Elements (hLINE) (189) (Fig. 5-4C). Mice injected with migratory as opposed to unsorted cells had a 2.5- and 4.5-fold increase in the amount of human DNA in their lung and liver, respectively (Fig. 5-5D-E). Metastasis was also detected in the axillary lymph nodes of 3/8 animals injected with migratory cells and only 2/8 animals injected with unsorted cells. Tissue samples from representative specimens were also processed for immunohistochemistry against human mitochondria, as well as hematoxylin and eosin staining (Fig. 5-5F). Representative images confirm the presence of metastatic human cells in the lung, liver, and lymph node of mice. Bone marrow was not intact after thin sectioning, and thus images are not shown. Taken together, these data indicate that while migratory and unsorted cell populations both form tumors that grow at similar rates, migratory cells have a markedly enhanced ability to form spontaneous metastases.

5.3.3 Characterization of phenotype and genotype of migratory cells. To understand what factors contribute to the increased metastatic potential of migratory cells, we first compared the phenotype of migratory and non-migratory MDA-MB-231 cells in MIND. Migratory cells move with higher velocity (net displacement over time), speed (average displacement over each 20min time interval) and persistence (net displacement over total distance traveled) (Fig. 5-6A-C) than
non-migratory cells. Migratory cells also spread over a larger area and have a higher aspect ratio, indicating their intrinsic ability to elongate in the direction in which they move (Fig. 5-6D-E). They also form more protrusions, as evidenced by their lower solidity (cell area/convex area) relative to non-migratory cells (Fig. 5-6F). Typically, these protrusions occur at the leading edge of the migratory cells, which may serve to probe the local microenvironment. Taken together, migratory cells are more elongated and more protrusive, and move faster and more persistently than non-migratory cells in vitro. These phenotypic differences may contribute to their enhanced metastatic potential in vivo.
Figure 5-6: Characterization of phenotype and genotype of migratory cells. (A) Velocity, (B) speed, (C) persistence, (D) spread area, (E) aspect ratio and (F) solidity of migratory and non-migratory MDA-MB-231 cells while in the feeder channel. Each data point represents the metric value for 1 cell averaged over its time in the channel for ≥50 cells from n=3 independent
experiments. Line and error bars represent mean±SEM. (G) Pathway analysis and (H) gene ontology annotation for DEGs in migratory cells. E.S., Enrichment Score. (I) Validation of select DEGs identified by RNA-seq using qPCR. Each data point represents the relative expression from 1 experiment. Column and error bars represent mean±SEM from $n=3$ independent experiments. *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$ by two-tailed Student’s t-test.
To elucidate potential gene expression differences contributing to the migratory cell’s increased motility in vitro and metastasis in vivo, we isolated RNA from equal numbers of migratory and unsorted MDA-MB-231 cells and performed genome-wide transcription analysis using RNA-sequencing (RNA-seq). RNA-seq analysis identified 1433 differentially expressed genes (DEGs, adjusted $p$-value <0.1) between the migratory and unsorted cells, consisting of 582 upregulated and 851 downregulated genes. Of note, these gene expression changes are not due to DNA damage induced by cell entry and migration inside the narrower branch channels, as evidenced by immunostaining showing similar levels of DNA damage marker phospho-H2A.X between migratory and non-migratory cells (Fig. 5-7). To understand the function of the DEGs, we performed pathway analysis and gene ontology clustering. We found that migratory cells had gene expression changes in multiple signaling pathways (Fig. 5-6G), including Ras/MAPK, PI3K-Akt, TNF, FoxO, and several pathways related to metabolism. Gene ontology clustering (Fig. 5-6H) revealed that migratory cells have differentially expressed genes relating to cell migration, regulation of apoptosis, metabolism, angiogenesis, and nitric oxide biosynthesis. Select genes from relevant pathways were verified for fold expression changes using qPCR (Fig. 5-6I). The PI3K pathway is activated in greater than 70% of patients with invasive breast cancer (129). While activating mutations in the canonical Ras/MAPK pathway occur at a lower rate (2-10%) in breast cancer (126), this pathway’s activity is linked to breast cancer metastasis, and its aberrant activity may be induced by overexpression of upstream receptor tyrosine kinases such as EGFR and Her2, which is common in breast cancer (127). Since the PI3K and Ras/MAPK pathways are implicated by the RNA-seq data and play a role in breast cancer progression, we sought to determine if activation of these two pathways was sufficient to confer metastatic ability in non-tumorigenic and non-metastatic cells in vivo.
Figure 5-7: Cell entry and migration inside the narrower branch channels do not induce DNA damage. (A-B) Migratory and (C-D) non-migratory MDA-MB-231 cells immunostained for phospho-H2A.X and the nucleus (Hoechst 33342) after migration in MIND. Scale bars, 5µm. (E) Number and (F) median area of phospho-H2A.X foci identified in the nucleus of migratory and non-migratory cells. P-values calculated by two-tailed Mann-Whitney test.
5.3.4 MIND predicts metastatic potential conferred by activation of PI3K and Ras/MAPK pathways in breast epithelial cells. The mammary epithelial cell line, MCF-10A, is non-tumorigenic and non-metastatic. Within this genetic background, we activated the PI3K pathway by knockout of the tumor suppressor, PTEN, activated the Ras/MAPK pathway through overexpression of activated KRAS(G12V), and created a double mutant cell line with both interventions (PTEN-/-KRAS(G12V)) (325). The metastatic potential of all four cell lines was first evaluated in MIND. The PTEN-/-KRAS(G12V) cell line displayed high percentages of migratory (24% at 13h) and Ki-67-positive cells (86%) (Fig. 5-8A-B). In comparison, parental MCF-10A cells and PTEN-/- cells exhibit low proportions of migratory (0% at 13h) and proliferating cells (33-40%). Importantly, this phenotypic behavior of PTEN-/- cells is not due to clonal selection, as three different clones display minimal migratory potential (Fig. 5-9). Interestingly, KRAS(G12V) cells exhibit similar motility (27% at 13h) but markedly lower proliferation (44%) than PTEN-/-KRAS(G12V) cells (Fig. 5-8A-B). The logistic regression formula established from our panel of breast cancer and breast epithelial cell lines (Table 5-1) predicts that only the combined activation of the PI3K and Ras/MAPK pathways will confer high metastatic potential on MCF-10A cells (Fig. 5-8C).
Figure 5-8: MIND predicts metastatic potential conferred by activation of PI3K and Ras/MAPK pathways in breast epithelial cells. (A) Percentage of migratory cells in PTEN-/-KRAS(G12V), KRAS(G12V), PTEN-/-, and parental MCF-10A cells. Data represent mean±SEM from \( n \geq 3 \) independent experiments. *, \( p < 0.05 \) for PTEN-/-KRAS(G12V) and KRAS(G12V) compared to MCF-10A and PTEN-/-; #, \( p < 0.05 \) for PTEN-/-KRAS(G12V) compared to KRAS(G12V) at 24h. \( P \)-values calculated by two-way ANOVA followed by Tukey’s multiple comparisons test. Dotted line designates 7% of migratory cells. (B) Percentage of Ki-67-positive cells in MIND. Each data point represents the percentage from 1 experiment. Column and error
bars represent mean±SEM n≥3 independent experiments. (C) Probability (%) of each cell line possessing high metastatic potential calculated using logistic regression and the percentages of migratory and Ki-67-positive cells. Baseline value (shown in white) for each heat map is set to the threshold value for each predictor (7% migratory cells and 55% Ki-67-positive cells). (D) Representative bioluminescent images of mice following tail vein injection with 10⁶ cells. Scale is shown to the right of each set of images. PTEN−/−-KRAS(G12V) cells form tumors (arrows) causing ethical endpoint before 20 weeks; the example shown for this condition corresponds to the third curve from the left in panel f. (E) Percentage of bioluminescent signal retained in the lung of each animal for 48h post-injection. For each time point, the background was subtracted from the peak signal and the difference was normalized to the initial value for that sample (n=5 per group). Data represent mean±SEM. **, p<0.01 for PTEN−/−-KRAS(G12V) compared to MCF-10A, PTEN−/− and KRAS(G12V) at 2 and 4h. P-values calculated by two-way ANOVA followed by Tukey’s multiple comparisons test. (F) Percentage bioluminescent signal in the lung for 20 weeks post-injection. Each curve represents 1 mouse (n=5 per group).
Figure 5-9: PTEN−/− clones consistently display low migratory potential. Percentage of migratory cells in different PTEN−/− clones compared to the parental population. Data represent mean±SEM from n≥3 independent experiments. Data from clone 20Pc5 is also shown in Fig. 5-8A-B and this clone was used for all other experiments.
To test this hypothesis, equal numbers ($10^6$) of parental, PTEN-/-, KRAS(G12V), and PTEN-/-KRAS(G12V) cells were injected into the tail vein of mice and monitored them with bioluminescent imaging (Fig. 5-8D). The tail vein injection model was selected because three of these cell lines do not form or rarely form subcutaneously implanted tumors (325), thus making comparison of their spontaneous metastasis from a primary tumor unrealistic. Following tail vein injection, the initial ability of cells to survive in the bloodstream and reattach in the lung capillaries was determined by imaging from 2 to 48h. PTEN-/-KRAS(G12V) cells exhibit higher bioluminescent signal in the lungs for 2-4h after injection, suggesting that they were able to firmly adhere within the lung capillaries (Fig. 5-8D-E). After 24-48h, the bioluminescent signal in the lung decreases to a similar level in each cell line, indicating that the elevated signal initially observed in the PTEN-/-KRAS(G12V) cells was dissipated by a combination of cell death, detachment and re-circulation within the blood stream, or migration from the blood vessel into surrounding tissue. Monitoring the cells for longer periods enabled us to assess their ability to extravasate, invade the surrounding tissue, survive, and proliferate. Each mouse injected with PTEN-/-KRAS(G12V) cells developed a tumor in the lung leading to an experimental endpoint between 9 and 14 weeks (Fig. 5-8D,F). No evidence of tumor formation was observed in mice injected with parental MCF-10A, PTEN-/-, nor KRAS(G12V) cells. Taken together, these results demonstrate that PTEN-/-KRAS(G12V) cells have high metastatic potential while MCF-10A, PTEN-/-, and KRAS(G12V) cells failed to form metastases in vivo. Furthermore, the MIND assay accurately predicted the metastatic potential of these cell lines.

5.3.5 MIND accurately predicts the metastatic potential of cells obtained from patient-derived xenografts. Patient-derived xenografts (PDX) are used as a model for breast cancer tumor growth and metastasis because of their ability to recapitulate the hallmarks of the patient’s disease (341, 342). We selected two well-characterized tumor specimens from triple-negative
breast cancer patients with metastatic disease (HCI-001 and HCI-002) (341) and expanded them as xenografts in mice. Since these specimens developed metastasis in the original patients and during their initial characterization as xenografts in mice, we expected that after quantifying the percentages of migratory and Ki-67-positive cells dissociated from the xenograft tumors using MIND, our logistic regression formula would calculate a high probability of metastasis. Indeed, dissociated PDX cells were able to migrate through the feeder channel and enter the branch channels (Fig. 5-10A). After each experiment, cells were fixed in the microfluidic device and immunostained for human mitochondria to ensure that the migratory cells strictly originated from the cancer patient and were not mouse stromal cells (Fig. 5-10C). Both HCI-001 and HCI-002 samples exceeded the threshold of migratory cells for each of the four optimal combinations of time and percentage of migratory cells (Fig. 5-10B,D). These samples also had high levels of Ki-67 cells (78% and 67%, respectively--Fig. 5-10E). Use of the migratory and proliferation indices in the logistic regression formula developed from the breast cancer and breast epithelial cell lines identifies both HCI-001 and HCI-002 as metastatic (probability=1). These experiments establish proof-of-principle that MIND can be used to predict the metastatic potential of clinically-relevant specimens.
Figure 5-10: MIND accurately predicts the metastatic potential of cells obtained from patient-derived xenografts. (A) Migratory HCI-002 cell entering left branch channel. (B) Percentage of migratory cells for PDX cell lines. Data represent mean±SEM from $n=5$ independent experiments. Dotted line designates 7% migratory cells. (C) Immunostaining for human mitochondria and nucleus performed after migration experiment. (D) Percentage of migratory cells in HCI-001 and HCI-002 after 13h. Each data point represents the percentage from 1 experiment for $n=5$ independent experiments. Column and error bars represent
mean±SEM. (E) Percentage of Ki-67-positive cells in MIND. Each data point represents the percentage from 1 experiment for $n\geq3$ independent experiments. Column and error bars represent mean±SEM.
5.3.6 MIND testing of therapeutic agents from ongoing clinical trials. Accumulating evidence suggests that breast cancer patients with the same molecular subtype may have differing responses to treatment (342-344). To date, no reliable indicator of response to a specific therapeutic regimen exists. Since MIND is capable of identifying highly migratory and proliferative cells that have enhanced metastatic potential, we hypothesized that it would be well suited for screening the effectiveness of potential therapeutics to inhibit the motility of these cells. As migratory cells have altered expression of numerous genes from the Ras/MAPK and PI3K pathways, we chose to evaluate inhibitors targeting these pathways that have potential for use in the clinic. The MEK1/2 inhibitor, trametinib, is FDA approved for use in melanomas harboring BRAF V600E or V600K gene mutations and is under evaluation for effectiveness in breast cancer in several active clinical trials. The PI3K inhibitor, BKM120, is also under evaluation in several clinical trials for use in breast cancer. A clinically relevant concentration of each drug was selected based on published pharmacokinetic data of each drug’s volume of distribution and patient dosage/target concentration (345, 346). For trametinib, 70nM was selected, and for BKM120, 1µM. Prior to testing in MIND, we confirmed that 24h treatment of either or both therapeutics did not significantly affect the percentage of viable cells compared to treatment with a vehicle control (Fig. 5-11A). Thus, any effect of the inhibitors on the percentage of migratory cells cannot be attributed to cell toxicity.
Figure 5-11: MIND testing of therapeutic agents from ongoing clinical trials. (A) Cell viability for SUM159, BT-549, and MDA-MB-231 cells treated trametinib for 24h relative to vehicle control treated cells. Select samples were also treated with BKM120 (1µM). Each data point represents the percentage from 1 experiment. Column and error bars represent mean±SEM of n≥3 independent experiments. (B) PI3K pathway genotype for SUM159, BT-549 and MDA-MB-231 cell lines and the effect of PI3K inhibition on their motility. (C) Mechanism for Raf activation during PI3K inhibition.
Trametinib treatment of three triple-negative breast cancer cell lines with high metastatic potential, SUM159, BT-549, and MDA-MB-231, was effective in reducing the percentage of migratory cells down to the threshold levels exhibited by cells with low metastatic potential (Fig. 5-12A-C). This pharmacological intervention also decreases the migration velocity of all three cell lines (Fig. 5-12D-F). The marked reduction in the percentage of migratory cells in response to trametinib treatment suggests its potential to reduce the risk of metastasis in patients bearing tumors with the characteristics of these breast cancer cell lines. Interestingly, pharmacological inhibition of PI3K yields different outcomes in these triple-negative breast cancer cell lines. Although BKM120 treatment reduces the percentage of migratory SUM159 and BT-549 cells compared to vehicle control (Fig. 5-12A-B), it significantly augments the migratory potential of MDA-MB-231 cells (Fig. 5-12C). In a clinical setting, an increase in the percentage of migratory cells is predicted to exacerbate the risk of metastasis. Similarly, BKM120 exerts divergent effects on the migration velocity of the aforementioned breast cancer cell lines (Fig. 5-12D-E). The combination of trametinib and BKM120 treatment failed to enhance the inhibitory effects of trametinib alone in all cell lines tested (Fig. 5-12A-F).
Figure 5-12: MIND testing of therapeutic agents from ongoing clinical trials. (A) Percentage of migratory SUM159, (B) BT-549 and (C) MDA-MB-231 cells after 13h during treatment with vehicle control, BKM120 (1µM), trametinib (70nM), or BKM120 and trametinib. Dotted line designates 7% of migratory cells. Each data point represents the percentage from 1 experiment. Column and error bars represent mean±SEM of n≥3 independent experiments. (D) Velocity of SUM159, (E) BT-549 and (F) MDA-MB-231 cells migrating in the feeder channel. P-values were calculated using one-way ANOVA followed by Tukey's multiple comparison test. NS, p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. (G) Representative western blot for pAkt in SUM159, BT-549, and MDA-MB-231 cells treated with vehicle control or BKM120 (1µM) for 24h. (H) Representative western blot for pErk in MDA-MB-231 cells serum-starved for 24h, treated with vehicle control or BKM120 (1µM) for 30min, then in select samples
stimulated with EGF (100nM) for the indicated time. Western blot experiments were repeated $n=3$ times. Full scans of the cropped blots appear in Fig. 5-13.
Further examination of the genotype of these three breast cancer cell lines (347) provided insights into their divergent responses to PI3K inhibition (Fig. 5-11B). SUM159 and BT-549 both harbor activating mutations in the PI3K pathway (H1047L mutation in the PI3K catalytic subunit or loss of PTEN, respectively). Inhibition of the overactive PI3K pathway in both of these cell lines decreased their percentage of migratory cells. In contrast, MDA-MB-231 cells have no activating mutations in this pathway. PI3K activity leads to the phosphorylation of the effector protein, Akt on serine 473 (pAkt). Western blotting indicates that while these three cell lines express similar levels of Akt, SUM159 and BT-549 both have significantly higher pAkt than MDA-MB-231 (Fig. 5-12G, Fig. 5-13A-C), in line with their activating mutations in this pathway. Addition of BKM120 decreases the level of pAkt in all three cell lines. It is established that Akt can inhibit Raf, reducing downstream signaling to MEK and ERKs (348). We thus hypothesized that inhibition of PI3K in MDA-MB-231 alleviates Akt inhibition of Raf, increasing downstream signaling in the Ras/MAPK pathway (Fig. 5-11C).
**Figure 5-13: PI3K and Ras/MAPK signaling in triple negative breast cancer cell lines.**

Representative western blot for (A) pAkt (Ser473), (B) Total Akt and (C) actin in MDA-MB-231, BT-549, and SUM159 cells treated with vehicle control or BKM120 (1µM) for 24h. Cropped versions of these scans appear in Fig. 5-12G. (D) Representative western blot for pErk and (E) total Erk in MDA-MB-231 cells serum-starved for 24h, treated with vehicle control or BKM120 (1µM) for 30min, then in select samples stimulated with EGF (100nM) for the indicated time. Horizontal lines denote separation between two membranes that were processed and imaged in parallel. Cropped versions of these scans appear in Fig. 5-12H. (F) Quantification of western blots for pErk and total Erk (representative images shown in Fig. 5-12H). Data represent the mean±SE of densitometry measurements from three independent experiments. *, p<0.05 for BKM + EGF compared to VC + EGF and VC; #, p<0.05 for VC + EGF compared to VC. NS,
$p \geq 0.05$. *P*-values calculated by two-way ANOVA followed by Tukey’s multiple comparisons test.
To test this hypothesis, we used western blotting to assess the phosphorylation levels of the Ras/MAPK pathway’s downstream effectors, Erk1/2 in response to EGF stimulation in the presence or absence of BKM120. EGF increases the levels of pErk in vehicle control-treated cells from 5-30 min, while at longer times (≥1h), the pErk level is similar to that of unstimulated cells (Fig. 5-12H, Fig. 5-13D-F). In line with our hypothesis, cells pre-treated with BKM120 and then stimulated with EGF displayed higher pErk levels than those of EGF-stimulated vehicle control cells from 5min to 1h (Fig. 5-12H, Fig. 5-13D-F); thus, BKM120 treatment not only increased the level of pErk at early time points, but also prolonged the duration of this signal beyond the time frame that the vehicle control treated cells showed activation. Taken together, these results demonstrate that inhibition of PI3K can increase Ras-mediated cell motility in MDA-MB-231, but not in SUM159 or BT-549 cells. Using the MIND assay, we herein demonstrate the potential to use a phenotypic test to rapidly screen the efficacy of therapeutics for reducing metastatic potential without the need for genetic testing and analysis to attempt to predict the response.

5.4 Discussion

To date, the diagnosis of metastatic potential in breast cancer involves the consideration of multiple independent parameters such as the grade, stage, and molecular subtype, which do not provide complete information on the expected outcome of a patient’s cancer. A second limitation to breast cancer treatment is the lack of a functional assay to evaluate the efficacy of novel therapeutic drugs on a patient-specific basis for personalized medicine. We herein developed a novel companion assay that provides information on the patient’s risk of metastasis by measuring both the motile and proliferative potentials of their cancerous cells, which are key properties for establishing metastatic colonies. The advantages of MIND include the physical isolation of highly migratory/highly proliferative cells, which allows subsequent detailed molecular and genetic characterization. MIND has been standardized by examining a large panel of normal-like breast
epithelial and cancer cell lines and validated using engineered cell lines with demonstrated metastatic potential and PDX specimens. MIND also offers a high-throughput technique for evaluating the potential efficacy of novel therapeutic regimens for precision medicine. This approach can yield results in hours, compared to the current gold standard of 4-12 months required for PDXs to grow in mice. Moreover, PDX are only successful in 28-37% of cases, leading to a significant loss of patient representation (341, 342). The ability to complete the assay within 24h enables prognostic clinical applications that would not be possible for a system that models the slow pace and complexity of in vivo metastasis. Furthermore, the assay evaluates the ability of potential therapeutics to inhibit the migration and/or proliferation of highly motile metastasis-initiating cells, rather than the growth of the unsorted tumor population.

We and others have demonstrated that metastatic cells are more migratory than non-metastatic ones (64, 323, 349). Our results indicate that the percentage of migratory cells correlates with the metastatic potential of breast cancer cell lines. Interestingly, we found the predictive power of the MIND motility assay was greater than that of the widely-used transwell-migration assay. In view of these findings, we conclude that entry into a confining pore itself is not sufficient for the prediction of metastatic potential but must be combined with a measure of directional persistence, and potentially with higher geometric complexity (decision making). Other conventional migration assays, such as wound-healing, were not compared to MIND due to their inability to isolate migratory cells for further study. We also extended our studies to clinically-relevant PDX specimens and found that our logistic regression formula accurately predicts their metastatic propensity. Assessing two metastatic PDX specimens provides a proof-of-principle for the use of MIND with clinically relevant cell preparations but lacks comparison to non-metastatic specimens. A limitation of the PDX model is that the tumor specimens that do grow tend to be the most aggressive (341), thereby limiting the availability of non-aggressive samples for comparison. MIND avoids this pitfall – as well as the selective pressures introduced
by long-term growth – by directly assessing tumor cells isolated from specimens without a requirement for growth either in vitro or as mouse xenografts. More comprehensive comparison of PDX specimens with low versus high metastatic potential will require the invention of new technologies that permit the reliable growth of a broader spectrum of patient tumors as PDX. Although some established prognostic markers are limited to specific breast cancer subtypes (28), MIND analyzes cell motility and proliferation, which are universal phenotypic features required by all metastatic cells independent of subtype and perhaps of tissue.

Combining our motility index with a clinically established proliferation index improves the predictive power of MIND by eliminating any false negative readings assessed by motility alone. Using normal breast epithelial and breast cancer cell lines, our accuracy is 100%. Ki-67 levels can be reproducibly assessed clinically by immunohistochemistry (IHC), which has already been shown to have prognostic value in breast cancer (338), and is recommended by the St. Gallen guidelines to help determine the molecular subtype. Furthermore, RNAseq reveals that Ki-67 (MKI67) is upregulated by migratory cells, affirming its role as a marker for poor prognosis. The percentage of Ki-67-positive cells was determined for tumor xenografts of MDA-MB-231, BT-549, HCI-001 (350) and HCI-002 (351) using immunohistochemistry (50%, 50%, 55% and 31%, respectively). These reported values are somewhat lower than our measurements in MIND. Percentages of Ki-67-positive cells are likely reduced in tumor samples due to the increased competition for limited nutrients and oxygen within the tumor microenvironment, as compared to the controlled media, pH, and oxygen available to isolated cancer cells in MIND. By using well-established breast cancer cell lines, we did not detect any differences in the proliferative index of migratory versus non-migratory cells. However, in patients’ samples, there may be a difference caused by competition in the tumor microenvironment. Thus, it will be important to specifically measure the proliferative index of migratory cells, which can only be done in situ using MIND.
Future studies with patient specimens will use the methods presented here to further validate the logistic regression coefficients developed for MIND.

MIND correctly predicts that the simultaneous activation of the PI3K and Ras/MAPK pathways enables breast epithelial cells to form metastases \textit{in vivo}. The tail vein injection model captures the latter stages of the metastatic cascade, including survival in the bloodstream, reattachment in lung capillaries, migration out of the blood vessel, migration/invasion in the lung tissue, persistent survival (dormancy), and recurrent growth. The MIND assay models the migration steps of this cascade, and assesses proliferation using Ki-67, which should predict recurrent growth. PTEN\textsuperscript{-/-}KRAS(G12V) cells possess high motile and proliferation indices, and only this cell line formed metastases in mice, in line with results from a murine model of prostate cancer (352). PTEN\textsuperscript{-/-}KRAS(G12V) cells were retained in the lung more efficiently at early time points, which was followed by the formation of large tumors by 9-14 weeks. Both PTEN\textsuperscript{-/-} and parental MCF-10A cells, which have low indices for migration and proliferation, fail to generate any tumors. Interestingly, KRAS(G12V) cells, although they have a high motile index, could not generate metastatic colonies, presumably due to their low proliferative index; this highlights the importance of evaluating the combined indices. Mice injected with parental, PTEN\textsuperscript{-/-}, or KRAS(G12V) cells were monitored for 1 year without signs of recurrence. The observations we have made in the tail vein model are in line with results from the subcutaneous injection of these cell lines (325), where PTEN\textsuperscript{-/-}KRAS(G12V) cells form rapidly growing tumors. In that study, PTEN\textsuperscript{-/-}KRAS(G12V) cells grown as xenografts in mice had increased Ki-67 levels compared to PTEN\textsuperscript{-/-} or KRAS(G12V) cells, which is in line with our results in MIND. Collectively, these data demonstrate the ability of the assessment of migration and proliferative potentials in MIND to predict the formation of metastatic colonies.

RNA-seq revealed that key pathways that are de-regulated in breast cancer, including Ras/MAPK and PI3K, contribute to enhanced migratory and metastatic potentials. Signaling
pathways and biological processes related to cell migration, proliferation, survival, and metabolism influence the phenotype of migratory cells. Since the PI3K and Ras/MAPK pathways play a role in breast cancer progression and metastasis, they have both been targeted by numerous therapeutics; however, cross talk between these pathways has limited the success of targeting either pathway individually, leading to the theory that simultaneous inhibition of these pathways is required for maximal inhibition of tumor growth and metastatic activity (128). This approach has had clinical success for anti-tumor activity in RAS or BRAF mutated non-small cell lung, ovarian, and pancreatic cancer (353), and is also under examination in advanced solid tumors (NCT01449058). It follows logically that for maximal metastatic potential, the activity of both pathways is required, which is confirmed by our functional and sequencing results.

Drugs are typically tested in patients based on the molecular subtype of breast cancer. However, the presence of different mutations among patients leads to varied responses to the same treatment. By using MIND to screen the efficacy of BKM120 at reducing metastatic potential, we discovered divergent responses in three triple-negative cell lines, highlighting the importance of our phenotypic assay to measure the potential efficacy of therapeutic drugs in a rapid, reliable and reproducible manner. We also deciphered the genetic factors responsible for the distinct responses of these breast cancer cell lines to the same treatment. PI3K, the target of BKM120, can regulate cell migration by altering the localization and activity of Rho GTPases (287). Cell lines with PI3K pathway activation (through PIK3CA mutation or PTEN loss) exhibited reduced motility after treatment with BKM120. Interestingly, in the cell line without PI3K pathway activation, MDA-MB-231, BKM120 enhanced cell motility. We revealed that this occurs due to a cross-talk mechanism between Akt and Raf, which resulted in increased Ras/MAPK signaling when BKM120 treatment reduced Akt-inhibition of Raf. This result is in line with the fact that knockdown of Akt1, a downstream effector of PI3K, potentiates invasion in breast epithelial and breast cancer cells (354). We also examined the effect of trametinib, a MEK
inhibitor, in these three triple-negative cell lines. This intervention consistently reduced the migratory potential of all three cell lines, in accordance with the well-studied roles of the Ras/MAPK pathway in supporting cell migration (355). Trametinib is currently being evaluated in clinical trials for breast cancer and advanced solid tumors, either alone or in combination with other inhibitors, including ones targeting the PI3K pathway. Prior work suggests that cell lines can develop dominant signaling pathways that inhibit the activity of other pathways through negative feedback loop mechanisms (356). Under this regime, MDA-MB-231 cells, which do not bear constitutive PI3K activation, would be resistant to PI3K inhibition, while sensitive to inhibition of the Ras/MAPK pathway. Indeed, Hoeflich et al demonstrated that MDA-MB-231 and other basal-like cell lines that depend on Ras/MAPK signaling (similar to that of Ras-transformed cell lines), are insensitive to PI3K inhibition while remaining sensitive to MEK inhibition (357). This study found that PTEN loss reduced sensitivity to MEK inhibition. Further, in prostate cancer cell lines that retain PTEN, it has been demonstrated that Akt-inhibition enhances Ras/MAPK signaling (358). Interestingly, although SUM159 and BT-549 cells have constitutive PI3K activity, they were still sensitive to MEK inhibition. This is likely due to sustained dependence on c-Raf/MEK/Erk activity, which can be activated as a result of the PI3K pathway in cell lines with constitutive activation of this pathway (359). Collectively, these results highlight the advantage of a phenotypic assay to evaluate the efficacy of therapeutics in the complex and varied genetic landscapes present in tumor populations.

MIND has the potential to be used in the clinical setting. When an area of abnormal tissue is detected in the body of a patient, a biopsy is performed to determine whether the lesion is benign or malignant. If the lesion is malignant, traditional pathology cannot predict the metastatic propensity of the primary tumor. Here MIND can be applied to rapidly distinguish between aggressive and non-aggressive cancers in order to provide information about a patient’s risk of metastasis and to potentially help generate individualized treatments. A small specimen of the
cancerous lesion can be obtained via biopsy or as part of a resection surgery. Mass-production of microfluidic devices with high quality assurance using thermoplastics or similar materials has been demonstrated by companies such as Optotrack and μFluidix. Lens-free imaging technology can be employed as an inexpensive way to monitor cell migration (323). This technology provides high cell-to-background contrast that is amenable to automated analysis of the time-lapse images. Preclinical validation using a cohort of prospective patients is required to confirm the logistic regression coefficients and probability threshold used to determine patient prognosis.

Given the promising performance of MIND in breast cancer, this functional assay might be relevant to other solid cancer types.

In conclusion, we have fabricated MIND to rapidly assess a cancer patient’s risk of developing metastasis. The functional assay combines the quantitation of cell migratory and proliferative propensities to provide an accurate assessment of the specimen’s metastatic potential. MIND requires a small sample, delivers rapid results, screens the effect of therapeutics on highly motile metastasis-initiating cells, and physically isolates these cells for further molecular and genetic characterization. Compared to unsorted cells, migratory cells isolated from MIND exhibit markedly increased metastatic propensity granted by the regulation of motility- and survival-related genes. Taken together, MIND can be used as a companion assay for clinical prediction of breast cancer metastasis and selection of effective therapeutic regimes.
Chapter 6

Future directions and concluding remarks

6.1 Introduction

Bioengineering models of cell motility have led to important advances in basic and translational science. These models have led to the discovery of new migration mechanisms (15, 70, 72) and revealed both the physical limits (252) and the remarkably plasticity of cancer cell migration (282). In this dissertation we used these tenants to model the intersection of physical cues in normal and cancer cell migration, and in this chapter discuss future directions for continuing this research. We have also applied bioengineering principles from the body of previous work to develop translational technology, a Microfluidic Invasion Network Device (MIND) for breast cancer diagnosis and precision medicine. We discuss the ongoing application of MIND to other cancer types. Future plans for the automation, commercialization and regulation of this technology are described. Finally, building upon basic science discoveries made in MIND, a “next-generation” high-throughput migration assay for potential basic science and translational applications is proposed.

6.2 Intersection of confined cell migration and shear stress

Intravasation plays an important role in many physio- and pathological processes (25). As discussed in Chapter 4, further work will be done to illicit the mechanisms by which migration fibroblast cells sense and respond to shear stresses, including the specific roles played by the activity of GTPases RhoA and Cdc42 in controlling migration direction in response to this stimulus. Further exploration of the mechanisms by which cancer cells become insensitive to
shear stress, and how they respond at higher shear rates will be pursued in order to understand how these cells successfully intravasate in vivo.

The advantage to our microfluidic model of confined cell migration followed by intravasation is its relatively high throughput compared to more complex models. This allows faster hypothesis testing in order to achieve mechanistic understanding of these nuanced processes. After gaining insight into the fundamental mechanisms, incorporation of other physical aspects of in vivo intravasation can be used to validate our mechanistic understanding. Seeding of a monolayer of endothelial cells on the wall of the microfluidic device would allow us to question if cells that push through the endothelial layer have altered sensitivity to shear flow. Monolayer integrity will be verified by imaging (via immunostaining or expression of live cell reporters) of E-cadherin junctions and ZO-1 tight junctions in the monolayer (174). As the monolayer will be oriented in the vertical plain relative to the glass slide, imaging techniques with high z-direction resolution will be employed, such as light-sheet microscopy or a Zeiss confocal microscope with Airyscan technology (both available at Johns Hopkins University imaging core facilities). Monolayer integrity will also be verified by its ability to segregate FITC-dextran solution to one side of the monolayer, such as used in (15).

We would also like to study the competition between chemotaxis (directed cell migration along a biochemical gradient) and shear stress, which in our system tends to turn fibroblasts away. Interstitial fluid flow driven outside of capillary vessels in vivo can create autologous gradients of growth factors and other nutrients that stimulate cell chemotaxis (130). As chemotactic gradients and shear stress likely intersect at during cell intravasation in vivo, the competition between these biochemical and physical stimuli would lead to deeper understanding of this process. The laminar flow of the larger channels of our microfluidic device are able to create finely-controlled chemotactic gradients along the microchannels (64), which would allow us to explore this question. Altogether, the ability to tune multiple physical parameters and
include interactions between multiple cell types and biochemical signals highlight the versatility of microfluidics in bioengineering.

6.3 Diagnosing cancer with your MIND: further clinical applications

Point-of-care devices for cancer diagnosis and precision medicine are the subject of many ongoing research efforts in order to improve patient outcomes. Challenges in deciphering the complex and diverse genetic drivers of many cancers have led researchers to instead design phenotypic assays with more direct outcomes for cancer diagnosis (27, 213, 214). This was the motivation of our development of the Microfluidic Invasion Network Device, and the results presented in this dissertation suggest that this technology should be applied to other solid cancers and further developed towards commercialization. In this section we discuss ongoing evaluations of using glioblastoma multiforme and pancreatic cancer.

Glioblastoma multiforme (GBM) is the most frequent and aggressive brain cancer in adults and accounts for ~15-20% of all brain cancers (360). Median survival for GBM patients is only ~14.6 months (360, 361). GBM’s aggressiveness is due to its high rate of proliferation and frequent invasion through the local brain parenchyma. MIND serves as a good model for this local brain microenvironment and can also assess the proliferation status of highly-migratory cells. In ongoing work, we obtain primary GBM cell lines established from patients undergoing resection surgery by Dr. Alfredo Quinones-Hinojosa (formerly at JHU and now in the Department of Neurosurgery at the Mayo Clinic in Jacksonville, FL). Using the experimental and analytical methods developed in this dissertation, the percentage of migratory cells, percentage of Ki-67 cells, and other measurements taken from MIND were assessed in GBM patient-derived samples. Using logistic regression to combine MIND measurements into a MIND composite score, we are able to predict patient overall survival and time to recurrence. The details of this
study are currently under consideration for publication at *Nature Biomedical Engineering* and select methods and results are presented here with permission.

6.3.1 *Glioblastoma cells and cell culture*. Patient-derived primary human GBM cells (Retrospective: GBM153, GBM276, GBM318, GBM496, GBM499, GBM501, GBM549, GBM609, GBM612, GBM626, GBM651, GBM653, GBM692, GBM714, GBM731, GBM832, GBM897, GBM940, GBM960, GBM965, GBM1049, GBM1298; Prospective: GBM1280, GBM1283, GBM1295, GBM1296) were isolated from primary tumor tissue samples of patients undergoing brain resection surgery for GBM at the Johns Hopkins Hospital with approval of the Institutional Review Board. All tumor samples were pathologically confirmed to be GBM. Tissue donors did not receive any treatment prior to surgery. The primary cells were isolated, purified and maintained through previously described methods that eliminate cross-contamination from other cell types and capable of maintaining the stemness and molecular characteristics of the original primary tumors (362). The primary GBM cells were grown as adherent cultures on tissue culture flasks pre-coated with laminin (Trevigen) at a density of 1 µg/cm² surface area diluted with PBS without magnesium and calcium for 3 h at 37°C. The culture media consisted of 1:1 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Invitrogen), Gem21 Neuroplex™ without vitamin A serum-free supplement (Gemini), 1× antibiotic/antimycotic solution (Sigma-Aldrich), 10 ng/ml of recombinant human fibroblast growth factor (Peprotech) and 20 ng/ml of recombinant human epidermal growth factor (Peprotech). Accutase solution (Sigma-Aldrich) was used to dissociate cells from the laminin-coated tissue culture flasks instead of trypsin.

6.3.2 Correlation between *in vitro* MIND and clinical data. The relationship between the migratory and proliferative measurements obtained with MIND to the clinical outcome of patients was examined by separating the samples into low and high survival groups based on the threshold
median GBM patient survival time of 14.6 months established by Stupp et al (361). Statistical difference between the MIND measurement metrics of the high versus low survival groups was assessed using a two-tailed student’s t-test. Additionally, Pearson’s correlation analysis was performed to assess the linear correlation between the MIND composite score and patient survivals (in months).

6.3.3 Combining the migratory and proliferative indices into a single composite score maximizes the prognosis performance of MIND. The % migratory, % narrow entry (of migratory cells that enter the narrower 3 µm-wide branch channel) and % Ki-67-positive migratory cells were combined into a composite MIND score (ranging from 0 to 1) using logistic regression (Fig. 6-1A). A composite MIND threshold score of 0.5 was used to stratify patient into high (>0.5, n=12) and low composite MIND score groups (<0.5, n=10). The differences in composite MIND score between the low and high survivors were magnified, with low survivors having a higher composite MIND score than the high survivors (Fig. 6-1B). The composite MIND score and GBM patient survival in months were linearly correlated with an R² value of 0.46 (Fig. 6-1C). This classification separates the two patient groups with a significant difference in median survival as assessed by Kaplan-Meier survival analysis (Fig. 6-1D). Importantly, the sensitivity, specificity, PPV and NPV and accuracy of the composite MIND score to correctly identify high and low survival patients was about 90%. Finally, the area under the curve of the receiver operating characteristic of the composite MIND score was 0.90/1.0, indicating that the composite MIND score is a very good binary discriminator (Fig. 6-1E).
Figure 6-1: Combining migratory and proliferative indices into a single composite score maximize the prognosis performance of MIND. (A) The values of composite MIND score computed with logistic regression by combining % highly migratory cells, % narrow entry and % highly migratory Ki67-positive cells as independent predictors. (B) Mean composite MIND score of low (red bar, n=13) versus high (blue bar, n=9) survivors. *** represents p<0.001 as assessed by unpaired student’s t-test. (C) Linear regression analysis of GBM patient survival against composite MIND score. Black solid line represents the best-fit line while black dotted line represents the 95% confidence interval. *** represents p<0.001. Pearson’s correlation was used to assess the significance between the variables. (D) Kaplan-Meier curve based on composite MIND score, comparing survival of the retrospective cohort as separated by high (>0.5, n=12) or
low (<0.5, n=10) composite MIND score. ** represents p<0.01 as assessed by two-tailed log-rank (Mantel-Cox) test. (E) Receiver operating characteristic curve of classifying GBM patients into high or low survivors based on composite MIND score. AUC was calculated to indicate the prognostic utility of composite MIND score in classifying GBM patient into high or low survivals. (F) Mean time to recurrence of low versus high % highly motile cells (1st panel), % narrow entry (2nd panel), % highly motile Ki67-positive cells (3rd panel), and composite MIND score (4th panel). * represents p<0.05 and ** represents p<0.01 as assessed by unpaired student’s t-test.
Aside from GBM patient survival, the composite MIND score can also be used to predict time to recurrence. GBM lines which were derived from patients with a high composite score, % narrow entry and % of highly motile Ki67-positive cells (Fig. 6-1F) had a significantly shorter time to recurrence. % highly motile cells alone also showed a trend in predicting time to recurrence thought not statistically significant (Fig. 6-1F).

In summary, by utilizing the methods developed for breast cancer, MIND was readily applied to make predictions in GBM with about 90% accuracy in this training set. Ongoing work in a prospective validation study has 100% accuracy for the four patients that have been assessed. These results indicate the promise of using a simple, phenotypic test for cancer diagnosis.

6.3.4 Application of MIND to pancreatic cancer cell lines. Pancreatic cancer remains one of the most deadly cancers due to its propensity to metastasize (125). MIND is well suited to examine the role of migration and proliferation to the metastatic progression of this deadly disease. Pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas. Initial screening of PDAC cell lines in MIND shows a wide range of migratory ability, which populations averaging from 5.8% to 39% migratory cells, with from 0% to 33% of these migratory cells choosing to enter the most confining 3 µm-wide branch channel (Fig. 6-2).
Figure 6-2: Screening of pancreatic ductal adenocarcinoma cell lines in MIND. (A) Percentage of migratory cells in MIND, defined as the proportion of cells that enter the feeder channel and go on to enter one of the branch channels. (B) Percentage of migratory cells that enter the narrow 3 µm-wide branch channel instead of the less-confining 10 µm-wide branch channel.
These data and that from additional PDAC cell lines will be used as a training set to establish the logistic regression parameters for migratory cells and narrow entry that best predict metastatic potential in PDAC, using the methods explained in Chapter 5. As in Chapter 5, the proportion of Ki-67-positive cells will also be assessed for prognostic ability. Validation of established predictive parameters will be performed using patient-derived xenograft specimens available at the National Cancer Institute’s Patient-Derived Models Repository.

6.4 Further MIND validation in breast cancer and Food and Drug Administration approval process.

The promising results generated using the MIND in this dissertation and in our ongoing work suggest that this technology could help improve patient outcomes in multiple cancers. To achieve this goal, further product development and commercialization need to be performed on the way to seeking approval from the Food and Drug Administration.

6.4.1 Automation and commercialization of MIND. Commercialization of MIND will require streamlining of its production and analysis pipelines for large-scale applications. Due to the utility of microfluidic devices such as MIND in many translation applications, multiple academic and commercial ventures are currently developing methods for the mass-production of microfluidic devices with high levels of quality assurance (363). A short list of these ventures includes the Center for Polymer Microfabrication (Singapore-MIT Alliance), the National Composites Centre (Bristol, United Kingdom), Optotrack, Inc. (North Carolina, United States), and µFluidix (Ontario, Canada). Collaborative or business partnerships with an entity such as these may be a viable route to mass production of MIND. Alternatively, ongoing work is evolving to develop microfluidic devices in more scalable materials than polydimethylsiloxane (PDMS), such as
thermoplastics and cyclic olefin copolymers (364), which could be evaluated in our lab as potential alternatives to PDMS.

MIND can be visualized using relatively inexpensive lens-free imaging options such as inline holography to monitor cell migration (323). This technology provides high cell-to-background contrast that is amenable to automated analysis of the time-lapse images. Automation of MIND data analysis using computer software will also be required for commercialization. Methods for automated tracking of cells are widely-available, as demonstrated by open-source software packages, such as TrackMate (365) and can be adapted to perform analysis of migratory cell behavior. Altogether, development of cost-effective fabrication, imaging, and analysis of MIND can be packaged for commercial distribution.

6.4.2 Further validation of MIND for use in breast cancer. Validation of MIND using additional clinical samples will be required in order to eventually gain approval for clinical use by the Food and Drug Administration (FDA). Initial studies should expand our use of patient-derived xenograft (PDX) specimens to increase the size of our current validation set using this model. In particular, the Welm lab’s PDX library includes a non-metastatic specimen, HCI-004, that should be compared to the metastatic specimens that we have already evaluated (341). Ideally these studies can be completed in parallel with MIND automation so that further validation studies utilize the commercial-ready product and require less user-input.

A prospective study utilizing human breast cancer patient specimens could be completed with the help of a willing clinician. MIND predictions can then be validated with actual clinical outcomes. Due to the relatively high survival times of breast cancer patients, this study will take many years and should be performed in parallel with other commercialization efforts.

6.4.3 Food and Drug Administration approval pathway for MIND. MIND falls under the regulation category of an in vitro diagnostic product (IVD) (366). IVDs are sorted into Class I, II,
or III based on the potential risk to patients. As MIND may eventually guide treatment decisions for high-risk diseases, it most likely falls into Class III. The first step towards gaining approval is to hold an IVD pre-submission consultation. This meeting with the FDA will be used to clarify our claims and present the supporting evidence and learn if or how we could better support our assertions in the eyes of the regulators.

Following the initial meeting, we will file for an investigation device exemption, which will allow us to distribute our investigational device for use in clinical trials in order to collect data on its safety and efficacy. Initially, these clinical trials would have to be prospective, with no treatment decisions made based on MIND data. During this phase we will collect clinical data needed to support MIND’s pre-market approval.

Pre-market approval is given to devices that demonstrate safety and efficacy in the clinic. For IVDs, the safety relates to the potential impact of false-negative and false-positive readings on patients, which is potentially high in the context of cancer diagnostics. The FDA reviews PMA submissions on a 180-day schedule. This process entails scrutinization of device manufacturing, clinical data sites, and premarket data supporting the claims of its efficacy. Safe and effective products are then cleared for marketing in the United States.

6.5 Next generation of high-throughput migration screening assays.

Increased experimental throughput leads to more rapid results. While working on this dissertation, our migration experiment throughput was limited by the availability of the microscope imaging systems, and the number of experiments that can be run simultaneously on one system. While the availability of these systems is beyond the scope of this dissertation, some thought has been given to ways that can increase the number of experiments that can be run simultaneously. The microfluidic platforms used herein are fabricated by bonding to standard sized microscope slides (25 mm x 75 mm). In Chapter 4, the device design supports bonding of 2
devices per slide, while in Chapter 5, the device design supports bonding of only 1. Decreasing the size of independent migration systems will increase the number of conditions that can be tested per experiment. In this section, several ways to achieve this goal are discussed and a specific application is given.

More than half the length of the smaller microfluidic devices used in Chapter 4 consists of media channels and their inlet/outlet wells. This configuration was elegantly designed to provide controlled chemotactic gradients (64); however, many relevant experiments do not require such a gradient, including all of the ones performed for this dissertation. As accumulating evidence supports that topographical features and contact guidance guide cell migration, migration in microchannels in the absence of a chemotactic gradient is still physiologically relevant (324). We suggest the elimination of these multiple media inlet/outlets in favor of a single inlet on one side of microchannels and a single outlet on the far side. As the length of migration microchannels used in this dissertation is 200-400 µm, orientation of the inlet and outlet wells of the independent migration devices parallel to the direction of the short side of the microscope slide will maximize the number of conditions per experiment.

The space between microchannels used herein is 50 µm, occupied by PDMS that is bonded to the glass slide. While some minimum width is required, we suggest that this width could be reduced to allow more channels per area, which is in line with the recommendations from published microfluidic design heuristics (63, 367). It is important to note that the limit of channels per area determines the number of channels that can be bound to one microscope slide, as well as the number of channels that fit into each field of view on a microscope. The limits of the barrier between microchannels for reproducible, functioning devices can be explored using cheaper photomasks generated by high-resolution printing on transparent plastic sheets. Evaluation of the integrity of bonded channels can be determined over time by filling with FITC-dextran and verification of its exclusion from areas occupied by the channels (15). Designs produced for ongoing work in our lab suggests that the minimum distance between channels can
be well below 50 µm, which should significantly increase the number of channels per device. Typical migration devices employed here have ~200 individual channels. Decreasing the area between these channels can be used to either fit more channels per individual device or create smaller devices with the same number of channels. In fact, 200, 3 µm-wide channels with 20 µm-wide barriers in between would occupy less than 0.5 cm in width.

Combining these two design suggestions we propose a device based on the schematic presented below (components not necessarily drawn to scale) (Fig. 6-3).
Figure 6-3: Compact microfluidic migration assay for high-throughput screening. (A) Drawing of a simple microfluidic chamber for migration assays. Circles represent inlet/outlet well that will be created with a hole-punch. Dark rectangles represent areas where PDMS pillars are bound to the glass slide to form microchannel walls. Cells can be added to the inlet well and allowed to fill the triangular space to a desired density during seeding. Spontaneous migration through the microchannels in the absence of a chemotactic gradient can then be observed. (B) The compact design and separation of areas that need to be hole-punched allows for multiple migration chambers to be bound to a single standard microscope slide. Chambers can be placed in close proximity to each other and can be molded into the same piece of PDMS. Each chamber is independent and can contain an individual experimental condition.
This design allows for many migration conditions to be evaluated in parallel. Commonly used comparisons may be different cell lines migrating under the same conditions, or one cell line migrating under different conditions, such as in response to multiple doses of a drug or on different extracellular matrix proteins or concentrations of proteins. The increased throughput of cell migration data acquisition can be combined with automated cell tracking analysis, such as employed in (365, 368). Work from this dissertation presents opportunities for future study using such a technology.

In Chapter 5, we used the MIND platform to sort highly migratory and metastatic cells from a bulk population of cancer cells. RNA sequencing of these cell populations led to the discovery of roughly 1,400 differentially expressed genes between the two populations. Further study into which of these genes contribute to the motile and proliferative behavior that contribute to the enhanced metastatic potential of this subpopulation could reveal prognostic markers and therapeutic targets to inhibit metastatic progression.

The size of this list of genes is daunting and likely prohibits the investigation of each individual gene using current technologies. The effective prioritization of candidates that are likely to play a universal or causal role in cancer metastasis should produce more rapid results.

We herein propose a pipeline where genes from the DEG list are evaluated by: (1) correlation of gene expression in breast cancer patients with their overall survival, (2) inhibition of gene function using pharmacological inhibitors, siRNA libraries, or sgRNA libraries (3) migration phenotype testing using a high-throughput migration assay. Genes that are simultaneously differentially expressed by highly-metastatic breast cancer cells, related to breast cancer patient prognosis, and related to breast cancer cell motility in vitro are highly likely to be involved in breast cancer metastasis.
6.6 Outlook

This dissertation presents applications for the use of microfluidic models in basic and translational science. Numerous other applications in these fields are proposed, and the potential development of personalized medicine techniques is particularly exciting. Clinical screening of cancer markers (369) and bacterial resistance (370) can also be accomplished using microfluidic platforms. The ability to combine spatial constraints, physical stimuli, and cell-cell interactions also lend to organ-on-a-chip applications for the in vitro modeling of tumor growth and metastasis (371), as well as physiological and pathophysiological function of human organs (372). Methods similar to those used in this dissertation could also be applied to the screening of immune cell migration to provide-patient specific updates on immune cell competence (365). Further validation and commercialization of the Microfluidic Invasion Network Device will hopefully improve the health and wellbeing of patients suffering from breast and other cancers.


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Christopher Lee Yankaskas was born on April 16, 1991 in Rockville, Maryland to Kurt and Brenda Yankaskas. He has an older brother, Michael and a younger sister, Julie. From less than a year of age, Chris and his family lived in Ijamsville, MD. Throughout his life, Chris was fortunate to have many great teachers that led him to where he is in his studies today. This pedigree began with his father, an engineer for the US Navy, and his mother, a businesswoman and educator who also homeschooled the children for 2 years. Following this, Chris joined the Magnet gifted and talented program at Urbana Elementary School, where he was invigorated by the academic challenge. In the fourth grade Chris learned about the Hippocratic Oath, and decided to become a medical doctor. This decision was also influenced by his family members who are medical professionals and researchers, James R. Yankaskas MD, Bonnie C. Yankaskas PhD, and Mary C. Yankaskas MD. With the help of his parents and uncle James – particularly for the paperwork – Chris completed a fifth-grade science project where he studied the effects of vitamin A and C supplementation on bean plants treated with the tumorigen, Agrobacterium tumefaciens, compared to a control plant; he would later repeat a simplified version of this experiment in a college lab class.

Chris’ early exposure to science led him to pursue it in other outlets. Through his father’s occupation and volunteer work, Chris became a volunteer for the International Human-Powered Submarine Races, an engineering competition where primarily college students design, build, and race non-water-tight submarines that are powered by (bike) pedal-driven propulsion systems and operated by pilots using SCUBA gear. Chris and his siblings performed organizational tasks, helped lay the underwater course, and operated underwater live video cameras that enabled spectators to observe the races. Chris and his brother also spent considerable time together
playing street and ice hockey growing up, as well as fighting with their sister (but they’re cool now).

Chris began to develop leadership and public speaking skills through his involvement in the Boy Scouts of America. In addition to the general fun of camping, kayaking, and the great outdoors, this organization offered many opportunities to hold leadership positions with increasing responsibility. These opportunities taught Chris organizational skills, public speaking, and management of other people. Chris became an Eagle Scout at the age of 14 with the completion of his Eagle Project, construction of a 28’ footbridge leading into the nature trails at Friends Meeting School, where his mother still works. During high school, Chris had the benefit of taking Advanced Placement courses in biology, chemistry, calculus (shout out to Mr. Arbaugh and MathFace), and physics. He greatly appreciates Dr. Lillard letting him skip the state-mandated biology course to enroll in AP Biology, and also for generally letting him sleep in class other than for the lecture on ATP production. Chris also fondly remembers George and Michele Shearer—his AP physics and AP chemistry teachers, respectively, for their dedication to science and good teaching. Michele was recognized as National Teacher of the Year in 2011, two years after Chris graduated high school. Her chemistry class was one of the principle reasons that Chris decided to major in Chemical Engineering.

In keeping with Chris’ lifelong goal of becoming a doctor and his heritage as a Maryland resident, he applied to attend Johns Hopkins University (JHU) as an undergraduate. He was sorely disappointed after being placed on the wait list, and eventually not admitted. Chris instead attended the University of Maryland, Baltimore County (UMBC). The small class size of the Chemical Engineering program at UMBC quickly knit together a close community of students in this major that greatly benefitted Chris during his time in the program, and still to this day through friendships and a profession network. The professors within this department were and still are passionate about teaching, accessible, and personable. After pestering Dr. Mark R. Marten for a whole semester to give him a research position in his lab, Chris began his research
career the summer after his freshman year. The Marten lab gave Chris an independent project developing and implementing semi-automated techniques to study the growth and morphology of filamentous fungi. Chris thanks Mark and Usha Sripathineni for their time and mentorship. It was also during this period that Chris became familiar with an open-source image analysis program called ImageJ; he little suspected how much of his graduate years would be spent using this program.

Chris grew to be passionate about chemical engineering and during his senior year worked as a teaching assistant for Dr. Taryn Bayles’ course on Chemical Engineering Analysis. He chose the bioengineering/biotechnology track and was fascinated by his biology classes, and Cell Biology in particular. It was Chris’ positive experience at UMBC and in ChemE/bioengineering in particular that led him to apply to PhD programs in Chemical and Biomolecular Engineering. He graduated from UMBC with a 4.0 g.p.a. but was not selected as Valedictorian due to excellent competition. In 2013, Chris was accepted as a PhD student and Schwarz Scholar at JHU, and finally got to go to Hopkins.
Chris Yankaskas
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EDUCATION

Johns Hopkins University | Baltimore, MD
2013 – 2019 | Ph.D., Chemical and Biomolecular Engineering

University of Maryland, Baltimore County | Baltimore, MD
2009 – 2013 | B.S., summa cum laude, Chemical Engineering: Biotechnology and Bioengineering Track

RESEARCH EXPERIENCE

Graduate Research Scientist | Laboratory of Dr. Konstantinos Konstantopoulos
2013 – Present | Department of Chemical & Biomolecular Engineering

- Developed a microfluidic assay to predict breast cancer patients’ risk of developing metastasis and to potentially determine which therapeutic options have the greatest ability to reduce this risk
  - Enables high-throughput screening of 50,000 cells from a tumor biopsy to measure their migratory and proliferative potentials, which are essential to the development of metastases
  - 100% accuracy for 29 established breast cancer and breast epithelial cell lines
  - Proof-of-principle demonstrated in patient-derived specimens
- Discovered genetic signature responsible for enhanced metastatic potential through isolation of small populations (≤ 500 cells) of highly motile cells, which have enhanced metastatic potential
- Mentored students in laboratory skills, literature comprehension, and experimental design
- Managed laboratory equipment, inventory control, and safety compliance for 20-person research team
- Introduced and instructed use of CRISPR/Cas9 gene editing technology to laboratory
- Investigated the role of mechanosensitive ion channels in sensing physical force during cell migration
- Optimized cell elastic modulus measurement using atomic force microscopy for Cell Reports publication, enabling subsequent application of technique to several other lab projects

Undergraduate Research Scientist | Laboratory of Dr. Mark R. Marten
2010 – 2013 | Department of Chemical, Biochemical & Environmental Engineering

- Created an undergraduate research team to study fungal growth and nutrient recycling via autophagy
- Trained and mentored graduate and undergraduate students on fungal cell culture, live cell microscopy, and quantitative image analysis
- Automated image analysis process using open source software to improve throughput and reduce cost
SKILLS AND TECHNIQUES

- Project management
- Written and oral communication
- Lithography and microfabrication of microfluidic devices
- Gene knockdown/knockout via lentiviral delivery of shRNA or CRISPR/Cas9 editing
- Fluorescence and live-cell microscopy
- Use of FRET, gene promoter-GFP, and optogenetic biosensors to study protein activity and gene expression
- RNA isolation and gene expression analysis via RNA-sequencing and quantitative PCR
- Protein expression analysis by Western Blotting
- Bacterial, fungal, and mammalian cell culture

PATENT


PUBLICATIONS

In preparation:
Yankaskas CL, Mistriotis P, Bera K, Konstantopoulos K (2019). Fibroblasts don’t go with the flow: shear stress tunes actomyosin contractility to regulate fibroblast migration and persistence.

Under consideration:

Under review:

Accepted:

Published:

CONFERENCE PRESENTATIONS


TEACHING EXPERIENCE

**Guest Lecturer | Cell Biology for Engineers**
2016 – 2019 | Instructors: Dr. Joy Yang and Dr. Rong Li, JHU
- Lectures: Cell Mechanical Properties, Cell Adhesion Under Shear Stress
- Enhanced student comprehension by presenting fundamental biological principles in the context of practical engineering applications
- Improved and evaluated students’ understanding of material by producing lecture notes, homework assignments, and examination questions

**Teaching Assistant | NanoBio Laboratory**
2015 – 2016 | Instructor: Dr. Peter Searson, JHU
- Instructed graduate students in the lithography of master molds for replica-molding of microfluidic devices and microcontact printing of protein for the geometric constraint of cell adhesion
- Promoted interdisciplinary study by providing theory, applications, and hands-on experience for utilizing engineering tools in the study of biological systems

**Teaching Assistant | Transport Phenomena 1**
2014 – 2015 | Instructor: Dr. Konstantinos Konstantopoulos, JHU
- Delivered review lectures twice a week on course material to improve students’ ability to define system and boundary conditions, simplify governing equations and integrate solutions
- Created and evaluated homework assignments and course examinations

**Teaching Assistant | Chemical Engineering Analysis**
2012 | Instructor: Dr. Taryn Bayles, UMBC
- Held weekly review sessions to improve students’ analytical approach to mass and energy balances
- Lectured on course content during instructor absence
COMMUNITY SERVICE

Chairman of the Board of Directors  | Hope for ULD (501(c)(3) non-profit)
2018 – Present  | Stow, OH

- Oversee the organization’s effort to raise funding for gene therapy evaluation in an animal model and subsequent clinical trial for Unverricht-Lundborg Disease (ULD), a rare, heritable, degenerative epilepsy
- Raised over $35,000 to date
- Foster relationships with legal, accounting, and healthcare professionals who assist in an advisory capacity
- Build website to increase public awareness of Unverricht-Lundborg Disease
- Manage and write code for donation portal and ensure compliance to General Data Protection Regulation (GDPR) guidelines required by the European Union

Production Director  | The Foundry Church
2013 – Present  | Baltimore, MD

- Oversee weekly transport, set-up, and operation of audio/visual, lighting, and recording equipment
- Recruit, train, and manage schedule for 15 volunteers
- Manage problem resolution in high-pressure situations
- Maintain equipment inventory and recommend technological improvements

Youth Mentor  | Thread – The New Social Fabric
2013 – 2015  | Baltimore, MD

- Mentored an at-risk high school student to facilitate academic advancement, community building, and career preparation
- Provided application assistance and helped to secure yearly opportunities for paid summer work through the Diversity and Academic Advancement Summer Institute (DAASI)
- Aided in college preparation through SAT practice, college visits, and application guidance

AWARDS

- 2019: Institute for NanoBioTechnology Student Research Forum Fall Final Competition, third place
- 2018: Graduate Student and Postdoctoral Fellow Research and Education Award, JHU Department of Chemical & Biomolecular Engineering
- 2018: ScienceMarket Early Adopter Award for Science Communication
- 2013 – Present: Schwarz Scholar, JHU Department of Chemical & Biomolecular Engineering
- 2012 – 2013: Tau Beta Pi Dodson Scholar
- 2011 – 2013: Consecutive UMBC Undergraduate Research Awards
- 2009 – 2013: UMBC Homestead Scholar
- 2009 – 2011: Maryland Higher Education Commission Distinguished Scholar Award