NEW APPROACHES FOR IDENTIFYING CRITICAL INTEGRIN-MEDIATED SIGNALING EVENTS IN DIRECTED CELL MIGRATION

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NEW APPROACHES FOR IDENTIFYING CRITICAL INTEGRIN-MEDIATED SIGNALING EVENTS IN DIRECTED CELL MIGRATION

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Dynamic regulation of cell adhesion on extracellular matrix (ECM) proteins plays vital roles in the establishment and maintenance of tissue structure. More specifically, the spatial coordination of cell-ECM adhesion foci, or focal contacts, influences direction-sensing in motile cells for a wide range of biological processes in metazoan life. Currently available evidence suggests that positive feedback loops between a few canonical signaling pathways focuses rapid turnover of focal contacts at the leading edge, where broad protrusions guide cells in one direction or another. For instance, Rho GTPases regulate the localization of specific actin cytoskeletal rearrangements, phosphatidylinositol 3-kinase (PI3K) -generated second messengers facilitate polarization of related signaling molecules, and focal adhesion kinase (FAK) may potentially act as a mediator of these distinct signaling pathways. This dissertation aims to elucidate mechanisms that coordinate these signaling pathways, with acknowledgement of the limitations of current methods in studying cell migration (Chapter 1).

To address questions relating to the role of certain signaling molecules in the maintenance of direction in migration, novel methods for depositing gradients of ECM molecules have been established, including one novel technique incorporating microfluidics, which is amenable to live cell tracking via standard microscopy (Chapter 2). This microfluidic device enables the relative impact of the overexpression of various key proteins to be examined. The subsequent study

suggests the role of FAK in facilitating maintained directional migration, also described as persistent migration (Chapter 3). These results suggest that FAK is a critical facilitator of direction-sensing and spatial regulation of focal contacts.

BIOGRAPHICAL SKETCH

Daniel Scott Rhoads was born in Sellersville, Pennsylvania, in November 1977 to Larry and Deborah Rhoads. Raised in Upper Hanover Township, he was educated by the Pennsylvania public school system in the Upper Perkiomen school district, where he excelled in mathematics and the sciences. Inspired by the popularity of the Human Genetics Project and the infectious enthusiasm of his high school biology teacher, Ms. Barbara Ryan, Daniel was attracted to molecular biology. Granted a small allowance by the district's Board of Education, he was introduced to DNA isolation from animal tissue, restriction enzyme digestions, and gel electrophoresis in an independent education project his junior year of high school. In the Fall of 1996, Daniel entered Lehigh University in Bethlehem, Pennsylvania, as a molecular biology major. In addition to his coursework at Lehigh University, he pursued an honors research project in Professor Vassie C. Ware's laboratory, studying ribosome synthesis in *Xenopus laevis* oocytes. He graduated from Lehigh in June 2000 with a Bachelor's degree and *cum laude* honors, and then began graduate studies in Cornell University's Field of Pharmacology in Ithaca, NY. After laboratory rotations, Daniel chose to apply his background in molecular biology to the pursuit of cancer pharmacology, as well as advancing the understanding of cellular signal transduction and the interaction of cells with their microenvironments, with Professor Jun-Lin Guan. It was the influence of Professor Guan that brought Daniel to the Nanobiotechnology Center, and methods of microfluidics and microfabrication for studying cell motility. Following completion of his doctoral dissertation, he plans to pursue postdoctoral research with Professor Mingming Wu at Cornell University, studying factors influencing the motility of neuronal stem cells and endothelial cells, in the Department of Chemical and Biomolecular Engineering.

To my grandparents, Russell and Margaret Freed, and Lloyd and Betty Rhoads – for reminding me of the value of education, and for their love, I would like to dedicate my dissertation to their memory.

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It has been a tremendous honor for me to have come so far as to defend my doctoral dissertation in the fascinating world of cell biology, but I could not have done it without the advice and assistance of others. First and foremost, I would like to thank my family for their patience as I spent all of these years as a student, and my parents in particular for their support of my interests in science. My grandparents also played a strong role in providing me the best education available. I greatly appreciate the intellectual guidance and support that my advisor, Dr. Jun-Lin Guan, has given me – he's always been positive, encouraging, and constructive. Dr. Guan has also offered me the opportunities that have made my research possible – funding, space and collaborations – and greatly enriched my graduate student experience. I would also like to thank my other committee members, Dr. Gregory Weiland and Dr. William Brown, who have challenged me to remember the "big picture" and examine scientific questions from multiple directions. Dr. Eberhard Bodenschatz, although no longer directly involved with research at Cornell, played an integral role in developing my thesis in its early stages. And this project would not have been possible without funding from the Nanobiotechnology Center, an NSF-funded science and technology center.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE:
INTEGRIN RECEPTORS AND DETERMINANTS OF POLARITY IN DIRECTED CELL MIGRATION

I. The Metazoan Microenvironment

a) Cell Migration in Biology

Regulation of cellular interactions in the metazoan microenvironment is an integral part of all aspects of biology, including embryonic development, physiological homeostasis in the adult, and dysregulation in disease. Each cell type modulates the common genetic program to its unique requirements, in order to navigate through the complex milieu of soluble and insoluble factors in vivo (Alberts et al, 2002; Hynes and Zhou, 2000; Ingber, 2002; Orr et al, 2006). In the developing embryo, mechanisms of migration mediate early stages such as gastrulation and neural tube formation, as well as later stages such as the formation of organs by epithelial cell types, vascular networks by endothelial cells, and the wiring of the brain by neurons. Much of migration in development and the establishment of tissue architecture depends strongly on cell-cell contacts, occurring by movement of sheets of cells according to a hard-wired genetic program (Gilbert, 2003; Affolter et al, 2003). Mechanisms of migration are also utilized in the adult organism, such as for wound healing of epidermal tissues, plastic reorganizations of the central nervous system, and immune surveillance by leukocytes, to broadly name a few roles for motility. These modes of migration tend to be less hard-wired, however, relying much more on the sensing of attractant cues from outside of the cell through adhesion receptors, receptor tyrosine kinases (RTKs), and G protein-coupled receptors (GPCRs). As such, cell migration in the adult animal is largely a phenomenon of single cells and independent of cell-cell contacts (Laughenburger & Horwitz, 1996; Ridley et al, 2003; Wehrle-Haller and Imhof, 2003; Vicente-Manzanares et al, 2005; Schwartz and Horwitz, 2006), and the loss of cell-cell contacts correlates with transition to a mesenchymal-like morphology and seemingly

stochastic migration of cells in neoplasms (Polette et al, 2004; Friedl, 2004; Yang et al, 2006).

As the defining material of connective tissues in the body, the extracellular matrix plays a vital role as a functionalized scaffolding, holding the tissues together in vivo, and is derived mainly from cells of mesodermal origin. ECM is composed of various proteoglycans and glycoproteins, such as collagens, elastins, laminins, fibronectin, fibrinogen, and vitronectin, which can be found in various proportions depending on the location in the body and to modulate the strength, elasticity, lubrication and biochemical signals to the tissues that require such properties.

b) Fibronectin as a tissue organizer

As mouse knockout studies have shown, fibronectin is a critical ECM component, and is required for formation and maintenance of the mesoderm, epithelial and endothelial cell monolayers that line the airways, gastrointestinal tract, and vascular system of higher organisms (George et al., 1993). Fibronectin null mice survive beyond gastrulation, but exhibit signs that although the mesoderm forms, it forms with significant defects. For instance, mutant embryos fail to develop certain mesodermally-derived structures (notochord, somites) and develop structures (heart, blood vessels) variably and abnormally, and these defects appear primarily responsible for embryonic lethality. Similar studies of fibronectin in *Xenopus* embryos indicate that polarized fibrils are required for guiding the migrating mesoderm during the formation of these processes following gastrulation. Winklbauer and Nagel, for instance, found that fibronectin fibrils act more as an orienting cue, rather than a haptoattractant, and were aligned along the axis of mesoderm migration (Winklbauer and Nagel, 1991).

In addition to providing a guide during movement of the mesoderm following gastrulation, fibronectin guides the branching morphogenesis preceding tissue and organ formation (Gumbiner, 1996; Sakai et al., 2003). Specifically, site-specific accumulation of fibronectin is essential for cleft formation during initiation of epithelial branching in submandibular salivary-gland epithelia, as well as in the developing lung and kidney, facilitated by directional fibronectin assembly and translocation in the developing cleft (Larsen et al., 2006). Larsen, Yamada and colleagues identified this role for directional fibronectin assembly in branching via washout and pulse-chase experiments, which revealed that older fibronectin accumulates at the base of the clefts and translocates inwards as a wedge, with newer fibronectin assembling behind.

Similarly, fibronectin performs a significant role in vascular development and morphogenesis (George et al., 1993; Sottile, 2004), and soluble fibronectin from plasma is deposited into tissue extracellular matrices by a regulated, cell-dependent process, that promotes growth, survival and migration of endothelial cells (Hynes, 1990; McDonald, 1988; Akiyama et al., 1989; Sechler & Schwarzbauer, 1997). In addition, both fibronectin deposition and its primary integrin receptor are upregulated in neo-vessels following treatment with angiogenic factors such as vascular endothelial growth factor (VEGF) (Kim et al., 2000), suggesting an important role for fibronectin cell-ECM interactions in angiogenesis.

A third major role for fibronectin as an extracellular matrix ligand in cell migration is that of tumor metastasis (Yamada, 2003; Hood & Cheresh, 2002; Ruoslahti, 1999). In tissue stroma, fibronectin and other ECM proteins provide adhesive signals, as well as a steric blockage of cell movement, to biochemically and mechanically restrict the movement of neoplastic cells from

their primary tumor sites. Following loss of E-Cadherin expression and cell-cell contacts, many tumor cells exhibit altered expression patterns for ECM receptors (Su et al., 2002), along with increased secretion of matrix metalloproteases, to remodel tissue matrices and invade the vascular system (Polette et al., 2004; Zaman et al., 2006). Collectively, these changes are described as the epithelial to mesenchymal transition (EMT) (Thiery, 2003; Gotzmann et al., 2004), and enable neoplastic cells to escape from ECM-restrictions on motility (Friedl et al., 1998; Cukierman et al., 2002; Zaman et al., 2006).

II. The Molecular Basis for Adhesion and Migration

a) Integrins anchor the cell

The cellular counterpart to ECM proteins such as fibronectin is the integrin family of heterodimeric cell surface receptors. Integrins are heterodimeric transmembrane glycoproteins, composed of alpha and beta subunits, with each subunit having a large extracellular ligand-binding domain, a single transmembrane region, and a short cytoplasmic tail (except in the case of beta4). Upon binding to specific ECM components, integrins are activated and clustered into regions of the plasma membrane termed focal contacts, or focal adhesions, which anchor the actin cytoskeleton to the underlying ECM (Hynes, 1992; Clark and Brugge 1995; Hynes, 2002). Comprised of greater than 20 different combinations of alpha/beta subunits, the integrin family of cell surface receptors has a wide variety of specificities, with some receptors binding to more than one ECM component and several ECM components being recognized by more than one integrin. In the ECM proteins fibronectin and vitronectin, the tripeptide sequence Arg-Gly-Asp (RGD) acts as a recognition

and binding site for a number of integrins, including alpha5beta1, alphaIIb beta3, and all or most of the alphav integrins (Ruoslahti and Pierschbacher, 1987), and this site acts synergistically with another peptide sequence Pro-His-Ser-Arg-Asn (PHSRN) to promote cell adhesion (Akiyama et al, 1995). The alpha5 beta1 integrin has received particular attention as the main fibronectin receptor in most cell types, and has been shown to promote focal contact function and play a role in several types of cancer (Su et al, 2002), as well as being required in normal mesodermal and neuronal development (Goh et al, 1997). Mouse knockout studies support the conclusion that alpha5 beta1 is the primary receptor for fibronectin; both fibronectin and beta1 null mutations are embryonic lethal with overlapping defects. And additionally, while the defects of alpha5-null embryos are less severe, and studies have shown that the alphav integrins can compensate marginally (George et al., 1993; Stephens et al., 1995; Yang and Hynes, 1996).

How alpha5 beta1-fibronectin interactions are functionally regulated is therefore of critical interest for understanding the basis of cell adherence in tissue development and maintenance, as well as defects of cellular behavior relating to tissue stroma (Hynes, 2002). Regulation of alpha5 beta1 and other integrin receptors is governed by two mechanisms: (i) clustering of integrins promotes increased avidity for ECM components, and recruits an intracellular plaque of proteins that establishes a strong mechanical linkage between the ECM and actin microfilaments; and (ii) modulation of the intrinsic affinity of integrin receptors through conformational changes generated at their C-terminal tails (Sanchez-Mateos et al., 1996). Regarding integrin clustering, a variety of studies in leukocytes have left little doubt that the association of multiple weak bonds is a common way of achieving significant molecular interactions (van

Kooyk and Figdor, 2000; Takaki et al., 2002), which probably also accounts for protein recruitment to nascent focal contacts.

The second issue, regarding integrin affinity modulation, has required more detailed examination of integrin receptor structure, however. Many integrins are not constitutively active, and can be expressed on the cell surface in an inactive state, in which they do not bind ligand or transduce signals across the cell membrane. Structural studies of integrin receptors have found that affinity states are principally controlled by the conformation of the C-terminal tails of the alpha and beta subunits, effectively opening and closing the ECM-binding site as a clam-like structure (Arnaout et al., 2005; Humphries et al., 2003).

Additional approaches using expression analysis of recombinant integrins has contributed to understanding of the mechanisms of integrin C-terminal cytoplasmic domains in affinity modulation (Hughes & Pfaff, 1998; Hynes, 2002). One such group of studies focus on the membrane-proximal sequences of alpha and beta subunit cytoplasmic domains, which share a similar organization of polar and nonpolar amino acids. Deletion of these sequences (GFFKR and LLv-iHDR, respectively) in either alpha or beta subunits activate integrins, independent of cell type and signaling pathway, suggesting that the association of these motifs represent a structural constraint on integrin activity (Crowe et al., 1994; O'Toole et al, 1994). Another region of the integrin cytoplasmic domains that performs a critical role is a well-conserved NPXY motif that is required for integrin activation. Calderwood et al. (1999, 2002) illustrated that the head region of talin interacted with the beta3 integrin tail at the NPXY motif via its phosphotyrosine-binding (PTB) domain, and activates integrin receptors by separating the alpha and beta cytoplasmic domains. Other

focal adhesion proteins such as FAK, may also activate integrin receptors in this manner via PTB-containing FERM domains (Liddington & Ginsberg, 2002).

Interestingly, the extracellular and cytoplasmic conformational shifts involved in integrin activation appear to be coupled in a bidirectional and reciprocal manner, best viewed in terms of an allosteric equilibrium or series of equilibria (Hynes, 2002; Arnaout et al., 2005). In this manner, binding of ECM ligand enhances separation of the cytoplasmic domains, allowing their interaction with focal adhesion structural and signaling proteins, and facilitating activation and recruitment of additional integrin receptors to the site of clustering, for most of the integrin receptor isoforms. Exceptions include platelet- and leukocyte-specific integrins, which maintain a much more stable inactive state most of the time, until they are triggered to attach to blood vessel walls and perform their physiological functions.

b) Focal contacts provide scaffolding

The clustering and dissociation of integrin cytoplasmic domains triggers the recruitment of a large number of membrane and cytosolic proteins to form submembrane plaques, termed focal contacts, and is dependent on associated actin organization (Schoenwaelder & Burridge, 1999; Zamir & Geiger, 2001; van der Flier & Sonnenberg, 2001). Integrin cytoplasmic domains are short and possess no catalytic activity or static structure, instead relying on the large array of focal contact proteins to provide those biochemical and mechanical functions. Among those cytoplasmic proteins directly interacting with integrin alpha subunits are several Ca^{2+}-binding proteins that appear to regulate integrin affinity states and/or act as protein folding chaperones (Coppolino et al., 1997; Lenter & Vestweber, 1994), Caveolin1-Fyn complexes that signal via the

MAPK cascade (Wary et al., 1998), and paxillin (as shown in the case of alpha4) (Liu et al., 1999).

Significantly greater interest has been focused on protein interactions with the beta subunit cytoplasmic tails, however. Cytoskeletal proteins such as talin, alpha-actinin, and filamin (Horwitz et al., 1986; Otey et al., 1990; Sharma et al., 1995) perform vital roles in assembling focal contact linkages with the cytoskeleton. Other protein interactions with the beta integrin tail are regulatory proteins, including focal adhesion kinase (FAK) and paxillin (Schaller et al., 1995), the serine/threonine integrin-linked kinase (ILK) (Hannigan et al., 1996), receptor for activated protein kinase C (Rack1) (Liliental & Chang, 1998), and protein kinase C (PKC) (Ng et al., 1999).

Besides direct interactions with integrin cytoplasmic domains, an increasingly extensive list of cytoplasmic molecules have been found to be localized to focal adhesions, many of which interact indirectly with integrins and/or actin microfilaments (Zamir & Geiger, 2001). Among these, vinculin appears to play a critical role in cell adhesion and motility, and is commonly used as a marker protein for focal contacts (Xu et al., 1998). Vinculin interacts with several junctional components such as actin and talin (Menkel et al., 1994), alpha-actinin (Kroemker et al., 1994), paxillin (Turner et al., 1990), vasodilator-stimulated phosphoprotein (VASP) (Brindle et al., 1996), and vinexin (Kioka et al., 1999). Other important focal contact proteins include gelsolin (Azuma et al., 1998), tensin (Lo et al., 1994a, b), radixin (Tsukita et al., 1989), zyxin (Beckerle, 1997), and profilin (Vojtek et al., 1991) This group of proteins can directly bind, cap, bundle, or nucleate actin filaments, establishing a framework or scaffolding that does the mechanical work of the focal contact: indirectly tethering the actin cytoskeleton to the clustered integrin receptors (Blanchoin et al., 2000).

There are also a variety of adaptor proteins that recruit additional structural and signaling proteins to focal contacts, among them being paxillin. Paxillin was initially identified as a tyrosine phosphorylated protein in v-src-transformed cells and found co-localized with focal contacts (Glenney & Zokas, 1989; Turner et al., 1990). Paxillin contains numerous potential protein-protein binding sites, including proline-rich motifs, SH2-binding sites, leucine-rich motifs, and four LIM domains (Salgia et al., 1995; Turner & Miller, 1994), and interacts with Src family SH3 domains (Weng et al, 1993), Crk SH2 domains (Schaller & Parsons, 1995), and Csk SH2 domain (Schaller and Parsons, 1995). Paxillin leucine-rich motifs interact with FAK and vinculin (Brown et al., 1998), and the LIM2 and LIM3 domains are important for targeting to focal contacts (Brown et al., 1996). Other studies have suggested that paxillin is involved in reorganization of the actin cytoskeleton through recruitment of p21 GTPase-activated kinase (PAK)/PAK-interacting exchange factor (PIX) complex (Turner et al., 1999), and in cell migration through recruitment of another adaptor protein, Crk (Schaller & Parsons, 1995; Petit et al., 2000; Webb et al., 2004).

p130Cas (Crk-associated substrate) is another well-studied focal contact adaptor protein, and was first identified as a highly tyrosine phosphorylated protein in cells expressing v-crk and v-src oncogenes (Sakai et al., 1994; Cary et al., 1998). As an adaptor protein, Cas contains multiple modification and protein-protein binding sites, including an N-terminal SH3 domain, several proline-rich regions, and a cluster of putative phosphotyrosine SH2-binding sites named the substrate domain (SD). Cas has been shown to interact with FAK through its SH3 domain, and is phosphorylated by FAK/Src complexes (Cary et al., 1998), which then recruits Crk via its SH2 domain to stimulate cell migration (Klemke et al., 1998).

Additional adaptor proteins involved in focal contact activities appear to expand upon the modular use of Src-homology domains (SH2/SH3), including Grb2, Grb7, Nck, and Crk. Of these, Grb2 and Nck are mainly implicated in cell proliferative signaling through Ras (Lowenstein et al., 1992; Lehmann et al., 1990), whereas Crk and Grb7 are implicated in cell motility (Klemke et al., 1998; Shen et al, 2002).

Next there are regulatory kinases in focal contacts, including the lipid kinase phosphatidylinositol 3-kinase (PI3K), serine/threonine kinases ILK and PKC, and tyrosine kinases such as FAK, Src, Fyn, Yes, and Csk. FAK in particular has received significant attention for its apparently central role in focal contact function, and will be discussed in greater detail in a later section of this chapter. Briefly, however, FAK's kinase activity and tyrosine phosphorylation state are both closely associated with integrin clustering and focal contact formation (Guan & Shalloway, 1992), and are vital in both the recruitment and regulation of a wide array of cytoskeletal-related proteins (Parsons, 2003). As a signaling molecule, FAK has been implicated in numerous adhesion-related behaviors, including spreading, migration, survival, and proliferation, and FAK-null embryos are embryonic lethal with severe defects in the mesoderm (Ilic et al., 1995). Due in large part to similarities with mouse knockouts of fibronectin and integrin subunits alpha5 and beta1, FAK has therefore received much attention in fibronectin-regulated behaviors in the mesoderm.

A truncated form of Src was first identified as the transforming protein of Rous sarcoma virus (RSV), and offered the first early hints of the importance of tyrosine phosphorylation in cell signaling as it related to proliferation and motility (Purchio et al., 1978). Related nonreceptor tyrosine kinases Fyn, and Yes join with Src to form the Src family of kinases (SFKs) (Thomas & Brugge,

1997), and these three proteins appear redundant in their cooperation with FAK in focal adhesion signaling (Jones et al., 2000). Csk appears to regulate SFKs via phosphorylation of their C-terminal inhibitory phosphotyrosine motifs (Howell & Cooper, 1994).

PKC includes a family of serine/threonine kinases with more than 10 members, varying in expression patterns, that are involved in integrin-dependent functions including adhesion, spreading and focal adhesion formation (Dekker and Parker, 1994; Defilippi et al., 1997). Talin, vinculin and paxillin have been identified as potential targets for PKC (De Nichilo & Yamada, 1996; Hyatt et al., 1994).

ILK is a serine/threonine kinase originally identified as an integrin beta1-interacting protein, involved in regulation of cell adhesion (Hannigan et al., 1996; Legate et al., 2006). As a scaffolding complex with PINCH and parvin, ILK appears to mediate an array of diverse signaling pathways with synergistic effects on actin cytoskeleton organization, adhesion stability, and transcriptional regulation. Indeed, deletion of the ILK gene in mice demonstrated that ILK is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation (Sakai et al., 2003).

PI3K is another important signaling molecule, and will be discussed in greater detail in a later section of this chapter. Briefly, however, PI3K phosphorylates the D3 position of the inositol ring of phosphatidylinositides (PtdIns) to generate the second messenger PtdIns 3,4,5-P_3 (PIP_3) (Insall & Weiner, 2001; Payrastre et al., 2001). PIP_3, in turn, recruits pleckstrin homology (PH)-domain containing proteins to the cell membrane, thereby spatially-regulating multiple signaling pathways.

And there are many more proteins that have been shown to colocalize with focal adhesions and/or directly interact with proteins having known

adhesion-related properties, and there are likely many more that have not yet been identified. As a result, the supramolecular complexity of focal contacts is daunting, and the network of signaling pathways involved in cell spreading, survival, proliferation and migration is a vastly challenging task. In the next section, however, I will describe the prevailing views as to how these many structural and signaling proteins coordinate to generate and regulate cell migration.

c) Migration is a dynamic, integrated process

The current model of cell migration as a dynamic process was described by Lauffenburger and Horwitz in 1996, and more recently by Ridley et al. (2003), and distilled the disparate studies on a variety of aspects of cell migration research into a single model. This model describes cell migration as a highly integrated multi-step process that coordinates protrusion of the membrane and actin cytoskeleton at the leading edge of motile cells, assembly of focal contacts with which to stabilize attachment in the direction of coordinated motion, disassembly and release of focal contacts at the cell rear, and contraction with which to drive the cell forward (Lauffenburger & Horwitz, 1996; Ridley et al., 2003; Li et al., 2005). While envisioned as a step-wise process, each "step" is occurring simultaneously, and provides mechanical feedback to the other aspects, thereby integrating and coordinating these four separate activities. At the core of this process is the dynamic regulation of adhesion and generation of propulsive force, and in subsequent sections we will discuss how motile cells regulate what direction they migrate in, but here we will discuss the basic biomechanics of cell motility.

In polarized migrating cells, active membrane processes, including broad, sheet-like lamellipodia and thin, spike-like filopodia, are constantly

extending and exploring around the cell front. Both of these structures are abundant in actin and actin-associated proteins, and their formation is tightly correlated with ongoing polymerization of actin filaments. Indeed, actin filaments are intrinsically polarized with fast-growing "barbed" or "plus" ends against the membrane, and slow-growing "pointed" or "minus" ends opposite the membrane, and this inherent polarity is used to drive membrane protrusion. In lamellipodia, filaments form a branching dendritic network, whereas in filopodia they are organized into long parallel bundles (Welch & Mullins, 2000). In both cases, actual membrane protrusion is driven by actin polymerization that is regulated by the actin-related protein (Arp) 2/3 complex and actin severing proteins, such as actin depolymerizing factor (ADF, or cofilin) and gelsolin. In lamellipodial actin networks, Arp2/3 complexes bind to the sides or tip of pre-existing actin filaments and nucleate filament branching. Activation of Arp2/3 complexes is localized by WASP/WAVE family members that are themselves localized to the cell membrane, and actual membrane protrusion is thought to occur not by filament elongation, but by an "elastic Brownian ratchet" mechanism (Welch & Mullins, 2000; Pollard & Borisy, 2003). Protrusion in filopodia is thought to occur by a filament treadmilling mechanism, in which actin filaments within a bundle elongate at their barbed ends towards the filopod tip and actin monomers are released at the pointed ends near the base. The distinct organization of these protrusions appear particularly well-suited to separate functions: localized activation of the Arp2/3 complex in lamellipodia can direct a broad movement of the cell body in a particular direction during migration, whereas the narrow filopodial extensions are well-adapted as exploratory sensors. For maintenance of movement begun by membrane protrusions such as lamellipodia to be sustained, extensions must be stabilized by the attachment of integrins and formation of focal contact

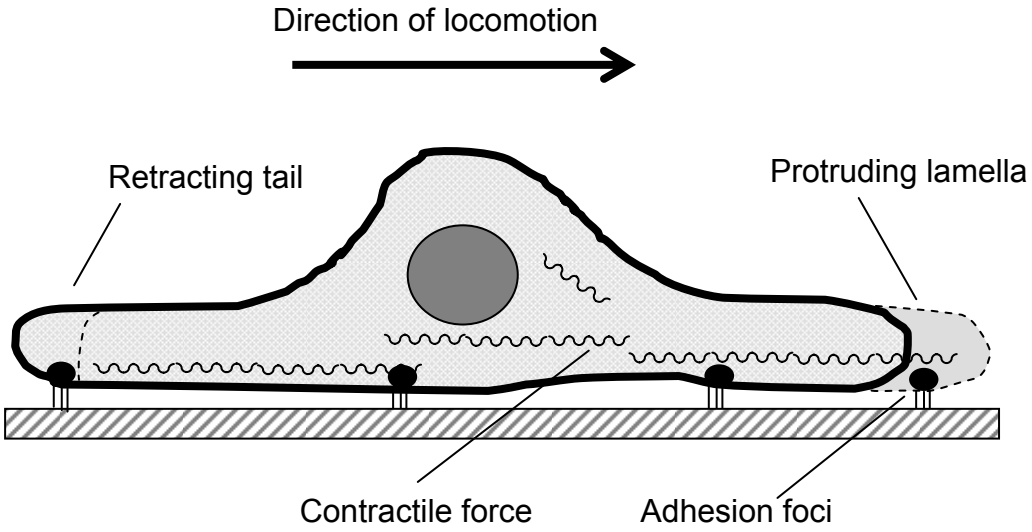

Figure 1.1. Cell migration is an integrated multi-step process. Cell migration involves the spatial coordination of four interrelated processes: Lamellipodial protrusions at the front of motile cells extend the leading edge; adhesion receptors form stable attachments to the extracellular matrix (ECM); old adhesions at the cell rear are disassembled; and molecular motors induce contraction of the actin cytoskeleton to produce traction forces.

structures. Integrin-mediated contact structures act as hand or feet with which to grasp or step forward, and activated integrins appear to preferentially localize to the leading edge, where new adhesions form (Kiosses et al., 2001). Adhesions or contacts come in different shapes and forms, referring to specific morphologies and methods of formation, including focal complexes, focal adhesions, fibrillar adhesions, and podosomes. Those at the front of migrating cells are small ($<1\mu m^2$) focal complexes, which are Rac-dependent, highly transient, and facilitate rapid migration by generation of strong propulsive traction forces (Nobes & Hall, 1995; Rottner et al., 1999; Beningo et al, 2001; Webb et al., 2002; Wehrle-Haller & Imhof, 2002). Assembly of focal complexes appears to occur via a sequential recruitment of "waves" of adhesion components, rather than the stabilization of preformed cytoskeletal complexes. As discussed above, Talin bidirectionally effects integrin affinity and protein recruitment to clustered receptors. FAK and tensin were identified early as components rapidly recruited to sites of integrin clustering via experiments using polystyrene beads (Miyamoto et al., 1995; Yamada & Miyamoto, 1995). Vinculin and alpha-actinin were also shown to be important in focal complex formation, but GFP-conjugating studies implicated a sequential recruitment of paxillin and alpha-actinin (Laukaitis et al., 2001). And recently paxillin has been shown to recruit a GIT1-PIX-PAK complex in a serine phosphorylation-dependent manner (Nayal et al., 2006).

Most focal complexes appear to cycle rapidly, persisting for only 20-30s as lamellipodia continue extending forward, as the focal complexes that were adjacent to the membrane become incorporated into the base of the dendritic actin network (Izzard, 1988; Galbraith et al., 2002). FAK-Src signaling has been strongly implicated in turnover of focal complexes at the base of

lamellipodia. In fibroblasts of knockout mice, the loss of FAK resulted in reduced migration rate, a reduced rate of spreading, and an increase in the number and size of peripherally localized adhesions (Ilic et al., 1995). Similarly, fibroblasts from mice lacking the Src family kinases Src, Yes and Fyn (SYF-null mice) have a decreased rate of spreading and reduced motility (Klinghoffer et al., 1999). Tyrosine phosphatases also have been implicated in adhesion turnover, including Ca^{2+}/calmodulin-activated protein phosphatase 2B (calcineurin), SHP-2, and PTP-PEST (Lawson & Maxfield, 1995; Yu et al, 1998; Angers-Loustau et al., 1999). Taken together, these studies underscore a central role for tyrosine phosphorylation in regulating protein-protein interactions critical to focal complex assembly and disassembly.

While most focal complexes turnover at the base of lamellipodia, some undergo Rho-induced enlargement to form robust focal adhesions (Rottner et al., 1999; Riveline et al., 2001; Wehrle-Haller & Imhof, 2002). Focal adhesions are larger (>1μm^2), elongated, take substantially longer to develop (~60min), are associated with stress fiber formation, and correlate with spreading and reduced motility. Differences in the molecular compositions between focal complexes and focal adhesions, aside from the involvement of Rho and some of Rho's effectors, are not well understood, nor are the mechanisms by which Rho is involved in this transition. The dynamics of Rac and Rho will be discussed at greater length in the next section.

As with disassembly of focal complexes at the base of lamellipodia, focal adhesions at the lagging edge or rear of migrating cells is required for locomotion, a process for which FAK/Src signaling appears to work as well. In particular, tyrosine kinase activity of Src appears required for the dissolution of focal adhesion components during cell migration (Fincham & Frame, 1998). While Rho activity promotes focal adhesion maturation, its inactivation appears

to loosen adhesions, eventually leading to cell rounding (Schoenwaelder & Burridge, 1999), and Rho inactivation can be achieved by serine phosphorylation by the cAMP-activated protein kinase A (PKA) (Lang et al., 1996). Also, the Cdc42/Rac effector PAK, which reduces myosin light chain (MLC) phosphorylation through phosphorylation of MLC kinase (MLCK), thus destabilizing actin stress fibres at focal adhesions (Sanders et al., 1999). And lastly, some studies have demonstrated that the tension generated by myosin-generated retraction forces can activate stretch-activated calcium channels at the rear of the cell (Lee et al., 1999), and activate the calcium-regulated phosphatase calcineurin and the calcium-regulated phosphatase calpain, which has the potential to cleave integrins, talin, vinculin and FAK (Hendey et al., 1992; Glading et al., 2002).

Coupling adhesion, protustion and actin organization is the myosin II-mediated contraction of the actin cytoskeleton (Schwartz & Horwitz, 2006). This model incorporates a regulated coupling of actomyosin contractility and functionalized adhesion foci, which both generates force sufficient for locomotion and sufficient attachment to the extracellular milieu to make use of this force. The degree of attachment is crucial however – too much adherence and cells prefer to remain spread and stationary, too little and the actomyosin-generated force fails to translate into forward-bound traction. This biphasic model of migration velocity was originally predicted by mathematics (DiMilla et al., 1991), and has since been supported experimentally by modulating ECM ligand density, integrin expression levels, and integrin-ECM binding affinity (DiMillia et al., 1993; Huttenlocher et al., 1996; Palecek et al., 1997). Inside the cell, however, this biphasic relationship of adhesion strength to degree of motility does not correlate with protrusion dynamics at the leading edge, but is instead correlated with changes in the organization and kinetics of the actin

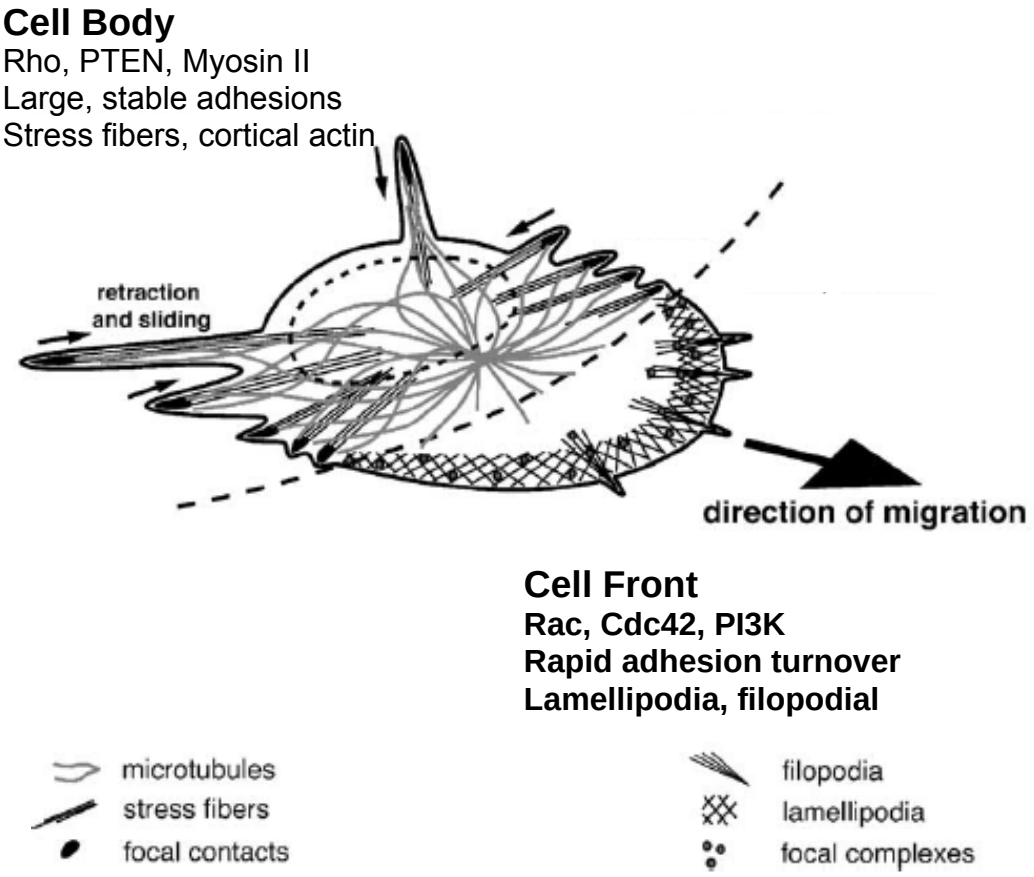

Figure 1.2. Focal adhesions and the actin cytoskeleton perform distinct functions in the front and back of motile cells. In the front, active actin polymerization and rapid turnover of adhesion foci at the base of lamellipodial extend the cell in the direction of locomotion, while at the rear, adhesions are degraded and the trailing edge retracted. Rac, Cdc42 and PI3K promote activities associated with the front, and Rho, PTEN, and Myosin II promote activities associated with the cell body. (Adapted from Wehrle-Haller & Imhof, 2003)

cytoskeleton, and is myosin II-dependent (Gupton & Waterman-Storer, 2006).

d) Rho GTPases as cytoskeletal organizers

One of the most heavily studied groups of regulators of actin cytoskeletal organization has been the Rho family of small GTPases, including the principle family members Rho, Rac, and Cdc42, for which over 50 effectors have been identified (Hall, 1998; Jaffe & Hall, 2005). Similar to other regulatory GTPases, they act as molecular switches cycling between an active GTP-bound state and an inactive GDP-bound state. This activity is influenced by (a) guanine nucleotide exchange factors (GEFs) that trigger the exchange of GDP for GTP to activate the switch (Schmidt & Hall, 2002); (b) GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity to inactivate the switch (Bernards, 2003); (c) guanine nucleotide dissociation inhibitors (GDIs), whose role appears to block spontaneous activation (Olofsson, 1999); and (d) covalent modifications, including direct phosphorylation and ubiquitination (Lang et al., 1996, Wang et al., 2003).

In their active states, Rho, Rac and Cdc42 perform distinct but related roles, including the assembly of contractile actin microfilaments, protrusive actin-rich lamellipodia, and protrusive actin-rich filopodia, respectively (Etienne-Manneville & Hall, 2002). These behaviors are mediated through coordination of actin-associated proteins, including the two major actin polymerization factors Arp2/3 and formin.

Although Cdc42 and Rac lead to morphologically distinct protrusions at the plasma membrane, they both utilize the Arp2/3 complex indirectly through members of the Wiskott-Aldrich syndrome protein (WASP) family. Cdc42, for instance, binds to N-WASP or the hemopoietic-specific WASP, to relieve an intra-molecular, auto-inhibitory interaction and expose a C-terminal Arp2/3

binding/activation site. However, this relatively simple model of Cdc42-mediated relief of N-WASP inhibition may not be the complete picture. Additional work suggests that much of cellular N-WASP is bound to WIP (WASP-interacting protein), which suppresses activation by Cdc42, providing an alternative mechanism for N-WASP inhibition. In this model, N-WASP may be both auto- and trans-inhibited, and activation of N-WASP may be assisted by another Cdc42 effector, Toca-1 (transducer of Cdc42-dependent actin assembly) (Ho et al., 2001; Martinez-Quiles et al., 2001; Ho et al., 2004). Rac, on the other hand, directs Arp2/3 activity through WAVEs, a group of proteins structurally-related to WASPs, but through an indirect interaction. How exactly Rac activates WAVEs is unclear, but a couple studies involving WAVE1 and WAVE2 suggest that Rac interacts directly with proteins that exist in a complex with WAVEs, and regulate their activity through localized recruitment and/or relief of inhibitory intra- or intermolecular interactions (Eden et al., 2002; Innocenti et al., 2004). Rho, conversely, induces actin polymerization through the formin family of proteins, including DRF (diaphanous-related formin), mDia1 (mammalian homologue of diaphanous), and possibly mDia2. GTP-bound Rho directly binds mDia1 and relieves an auto-inhibitory interaction, allowing binding to the barbed end of actin microfilaments via the FH2 domain of mDia1. mDia1 can then facilitate the delivery of actin monomers to the polymerizing microfilament end via an interaction with its FH1 domain and actin/profilin complexes (Zigmond et al., 2004).

Rho, Rac and Cdc42 perform multiple other functions that are critical to actin's roles in migration and coordinating direction, however. ADF/cofilin, for example, increases uncapped barbed ends, creating actin polymerization sites, and is required for productive membrane protrusions (Dawe et al., 2003; Ghosh et al., 2004). Cofilin also participates in filament disassembly, and can be

inactivated by phosphorylation by LIM kinases (LIMK), which are in turn activated by the PAK family of Rac/Cdc42-dependent kinases and by the Rho effector Rho kinase (ROCK) (Dawe et al., 2003; Ohashi et al., 2000). It is unclear how, but it appears that the strict spatial regulation of cofilin activity plays a critical role in protrusion dynamics. ROCK further has been identified in assembly of actomyosin contractile elements by phosphorylation of myosin light chain (MLC) phosphatase, which in turn promotes the actin microfilament cross-linking activity of myosin II (Riento & Ridley, 2003).

Rho family GTPases have also been demonstrated to influence microtubule (MT) dynamics and polarized alignment of the MT organizing center (MTOC). MT regulation is significant in directional migration, due to the abilities of MTs to target focal adhesions and accelerate their turnover (Krylyshkina et al., 1999), control local Rac and Rho activation (Ren et al., 1999; Waterman-Storer et al., 1999), and direct trafficking of Golgi-derived membrane vesicles to the leading edge to promote extension (Bershadskey & Futerman, 1994). Alternatively, MTs are stabilized at the leading edge by two known mechanisms. First, the Cdc42/Rac effector PAK phosphorylates a stathmin, thereby inhibiting MT destabilization (Daub et al., 2001; Wittman et al., 2004). Secondly, the Rho effector mDia1 is activated preferentially at the leading edge (Palazzo et al., 2001; Destaing et al., 2005). Thus, the current model for MT and Rho/Rac dynamics is that since Rac is activated transiently by new integrin binding, MTs should be selectively stabilized toward the front of the cell, where they further enhance focal contact turnover to favor new adhesion formation and deliver membrane components.

And lastly, with regards to polarized and directed cytoskeletal regulation by Rho GTPases, several studies suggest a positive feedback loop between Cdc42/Rac and phosphatidylinositol-3 kinase (PI3K) in neutrophils (Fukata et

al, 2003; Srinivasan et al., 2003). How Cdc42 and Rac promote activity of PI3K is unclear, but both the regulatory (p85) and catalytic (p110) subunits of PI3K bind GTPγS-bound Rac1 and Cdc42, and PI3K activity is enhanced by the GTPγS-bound GTPases (Zheng et al., 1994). How PI3K functions upstream of Rac and Cdc42, however, will be discussed in the following section.

e) Phosphoinositides as polarizing second messengers

Coming largely from studies in the slime mold *Dictyostelium discoideum* and in mammalian leukocytes, the role of phosphatidylinositol (PtdIns) lipids as second messengers for cellular signaling has gained widespread appreciation. During cell migration, these phospholipid second messengers effectively label the leading edge of cells, establishing the direction in which eukaryotic cells face. Their localization is regulated by lipid kinases such as phosphatidylinositol-3 kinase (PI3K) and its lipid phosphatase counterpart PTEN (Chung et al., 2001a; Funamoto et al., 2002; Iijima & Devreotes, 2002; Mañes et al., 2005).

The importance of PI3K phosphatidylinositol products PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 as key second messengers in cellular polarization was realized by recognition that pleckstrin-homology (PH) domain-containing proteins are recruited selectively to the leading edge membrane after exposure of moving cells to chemoattractant stimuli (Lemmon et al., 2002; Merlot & Firtel, 2003). The PH domains of these proteins bind to PI3K products in vitro, and their membrane localization in vivo can be disrupted by either critical point mutations that disrupt in vitro binding to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, deletion of PI3K, or inhibition of PI3K by LY294002 and Wortmannin (Dowler et al., 2000; Fukuda et al., 1996; Funamoto et al., 2001; Meili et al., 1999, 2000; Rickert et al., 2000; Salim et al., 1996; Servant et al., 2000). Further,

observations relating deletion of Class I PI3K isoforms to defective cell polarization and directed migration, and the roles of PH domain-containing proteins in mediating functions related to migration, strongly relate PI3K activity to directed migration (Chung et al., 2001b; Funamoto et al., 2001).

Many cytoskeletal proteins and proteins that regulate the cytoskeleton contain PH domains. One prominent group of PH domain-containing proteins that influences the cytoskeleton are the Dbl family of GEFs, that mediate activation of Rac/Rho family small GTPases by catalyzing the exchange of bound GDP for GTP (Whitehead et al., 1997; Rossman et al., 2005). Every Dbl family protein contains a Dbl homology (DH) domain responsible for its GEF activity, which is always immediately followed by a PH domain. Ligand binding to the PH domain is thought to regulate DH domain activity. In the case of Vav-1, it has been proposed that PI 3-kinase products bind to the PH domain and enhance the ability of the DH domain to activate Rac/Rho GTPases. Supporting this hypothesis, high concentrations of PtdIns(3,4,5)P_3 were reported to enhance Vav-1 exchange activity in vitro, but PtdIns(4,5)P_2 inhibits Vav-1 mutant with constitutive in vivo exchange activity (Ma et al., 1998). The resulting model suggests that D3-PtdIns, but not other inositols, somehow relieves an auto-inhibition of DH domain activity by PH domains. Similarly, the PH domain of Sos was reported to have an inhibitory effect upon its DH domain in vivo, and this could be relieved by deletion of the PH domain (Nimnual et al., 1998).

f) FAK as the central coordinator

The non-receptor protein tyrosine kinase FAK appears to participate in a remarkable number of the above-mentioned events linked to cell motility and polarity, and thus directionality (Mitra et al., 2005; Moissoglu & Schwartz,

2006). Cells lacking FAK have severe defects in both movement and polarization (Ilic et al., 1995), and FAK overexpression induces highly persistent directional motility (Gu et al., 1999). The role of FAK in directed motility is unclear, however, and it has been suggested to promote spatial regulation of focal adhesion turnover through several of its effectors. For instance, FAK phosphorylation of p130Cas (Cary et al., 1998) and paxillin (Schaller & Parsons, 1995), as well as recruitment of Src (Schaller et al., 1994) and PI3K (Reiske et al., 1999), have been implicated in focal adhesion turnover and migration. Which of these serves as the primary means of spatial regulation of focal adhesion status remains an open question, however, and indeed, the reality may be a complex combination of these protein-protein interactions in adhesion dynamics.

FAK activation is mediated by phosphorylation at multiple sites (Parsons, 2003; Mitra et al., 2005). Tyr^{397}, the major site, is stably phosphorylated in adherent cells and binds the SH2 domains of both Src and PI3K, among other proteins (Schaller et al., 1994; Xing et al., 1994; Chen et al., 1996). Tyr^{397} phosphorylation appears to disrupt binding between FAK's N-terminal FERM domain and its kinase domain, which relieves an intramolecular inhibition (Cooper et al., 2003; Cohen & Guan, 2005). This finding is in agreement with several observations. First, using chimeras between the interleukin-2 (IL-2) receptor extracellular and transmembrane domains fused with the integrin beta1 cytoplasmic tail, clustering of this chimeric receptor was found sufficient to induce FAK phosphorylation (Akiyama et al., 1994). Second, integrin beta1 tail mutations were identified that still allowed FAK recruitment to focal adhesions, but failed to induce FAK activation (Wennerberg et al., 2000). And third, induction of dimerization of a FAK/gyrase B chimera was shown to lead to increased FAK phosphorylation,

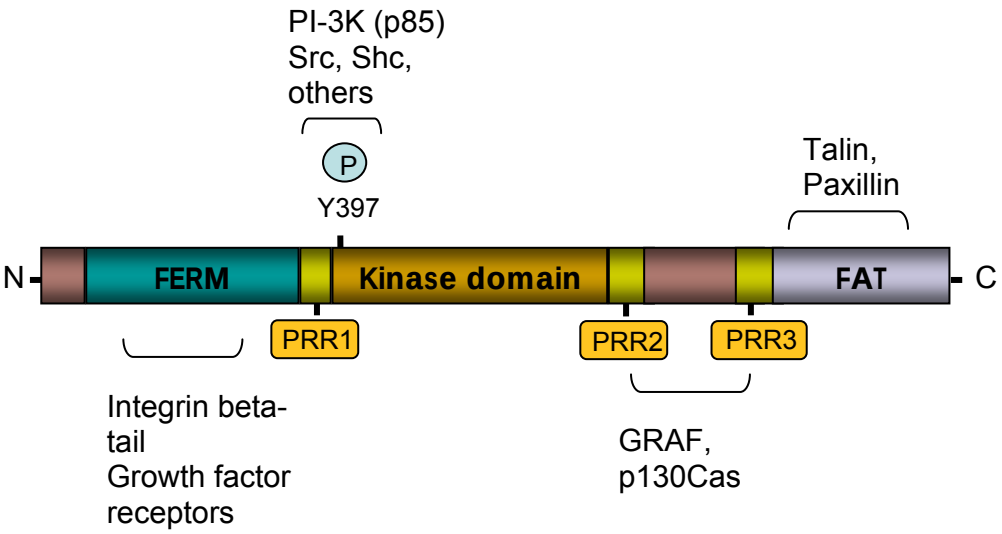

Figure 1.3. Focal Adhesion Kinase (FAK) domains and interactions. FAK contains an N-terminal FERM (protein 4.1, ezrin, radixin, moesin) homology domain, a central kinase domain, three proline-rich regions (PRR), and a C-terminal focal adhesion targeting (FAT) domain. FAT has been shown to be necessary for recruitment of FAK to focal contacts, FERM for relief of auto-inhibition of FAK catalytic activity, pY397 for full kinase activity and recruitment of key SH2 domain-containing proteins, and proline-rich regions for recruitment of adaptor signaling molecules.

suggestive of intermolecular transphosphorylation of FAK in FAK activation (Toutant et al., 2002). Collectively these data suggest that FAK activity is regulated by recruitment to sites of integrin alpha5 beta1 clustering, where intramolecular inhibition of FAK is relaxed, and some combination of auto- and trans-phosphorylation enhances the kinase activity of FAK, as well as increasing the accessibility of FAK to other signaling and scaffolding proteins.

As integrin clustering occurs primarily at the leading edge of motile cells, this activity at the front of the cell appears to provide the spatial regulation. FAK, in turn, appears to mediate multiple cytoskeletal processes, and thus focal contact stability via many focal contact proteins as described previously – Rho GTPases and PI3K as canonical examples. These signaling processes appear to function as a positive feedback loop, directing lamellipodia, and thus formation of nascent integrin adhesion complexes. Furthermore, FAK appears to be at a signal transduction crossroads, integrating chemotactic cues from growth factor receptors (Comoglio et al., 2003), G protein-coupled receptors (Hauck et al., 2000), cadherins (Avizienyte & Frame, 2005), and perhaps more. In such a way, FAK activity is influenced by a variety of biochemical events in the cell, thereby modulating the polarized activities of cell-ECM contacts in turn.

III. Innovative Approaches to Studying Cell Migration

a) Methodologies for Studying Directed Cell Migration

As cell migration is such a highly integrated and dynamic process, its study requires methodological approaches capable of teasing apart biochemical events both spatially and temporally. Over the years, a number of such methods have been conceived and developed (Guan, 2005). Among the most

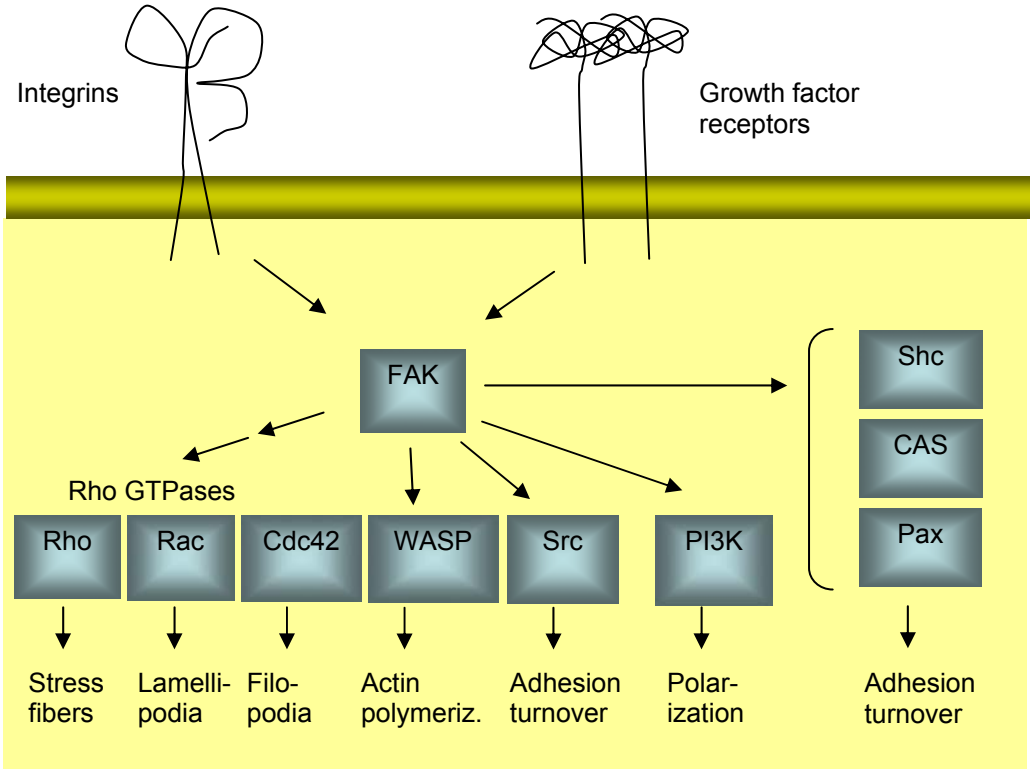

Figure 1.4. Focal adhesion kinase (FAK) integrates multiple signaling pathways through an array of outputs. FAK is activated by integrins, growth factors and other receptors during migration, and functions as a receptor-proximal regulator of cell motility. FAK indirectly influences the Rho-family GTPases (Rho, Rac and Cdc42) to regulate local actin organization. FAK can also directly regulate the localized signaling of WASP, Src and PI3K, and the structural functions of Shc, p130CAS and Paxillin, to name a few of the more prominent FAK effectors.

extensively-used methods are the Boyden chamber assay (Boyden, 1962) and wound-healing assays (Todaro et al., 1965). Each of these techniques has their strengths and weaknesses, and they continue to be added to by novel approaches. These classic and developing approaches to studying cell migration will be the topic of the remainder of this chapter.

One of the earliest methods developed for studying cell migration was the Boyden chamber, introduced in the early 1960's for the analysis of leukocyte chemotaxis (Chen, 2005). Also referred to as filter membrane or trans-well migration assay, it involves measurement of trans-well cell movement through a microporous membrane, as a result of chemotactic agents. Boyden chamber assays benefit from its simplicity, working with only a semi-permeable barrier and an attractant, and the results from it are equally simple – an enumeration of the relative degree of chemoattraction under differing conditions. For its simplicity and utility, the Boyden chamber is first among migration assays, but it does have its limitations. The Boyden chamber does not study cell migration in action per se, and does not involve the observation of changes in cell morphology or intracellular dynamics. As the toolkit of molecular biology has advanced in recent decades, migration assays were required which allowed imaging of motile cells, both in terms of time-lapse and spatial resolution of cellular processes.

At about the same time, the wound healing assay was also introduced in the 1960's, and mimicking in vitro cell migration during wound healing in vivo. This method is particularly well-suited for probing the roles of cell-cell interactions in sheet-like patterns of migration, such that cells may sense the loss of cell-cell contacts or the loss of proximity to adjacent cells, and thus migrate to close the wound (Rodriguez et al., 2005). The wound-healing assay does not examine the chemotactic sensing of and response to extracellular

attractants, however, and is not directly relevant to many deterministic cell migration processes in vivo, including pathogen surveillance by immune cells, tissue morphogenesis, angiogenesis, or intravasation of tumor cells. As such, while wound-healing assays are amenable to imaging of intracellular actions, their utility in understanding chemotaxis or haptotaxis is limited.

As the modern molecular biology era unfolded over twenty years ago, new experimental techniques incorporating chemoattractant gradients and observation of intracellular dynamics were developed. The prototype for such approaches was the Zigmond chamber, which allowed for the direct observation of slowly moving cells in a concentration gradient over longer periods of time (Zigmond & Hirsch, 1973). Later, this was adapted into the Dunn chamber, which afforded improved optical clarity, greater accuracy in characterizing chemoattractant gradients, and longer stability of concentration gradients for extended observations of chemotactic cells (Zicha et al., 1991; Dunn & Zicha, 1993). Essentially, the Dunn chamber consists of two concentric circular wells ground into the face of a glass slide, with an annular ridge separating the two wells. A concentration gradient of chemoattractant forms by diffusion across the ridge, from the outer well to the inner well. Cells seeded onto a coverslip are inverted and placed over the chamber, and cells exposed to the gradient above the ridge are viewed during chemotaxis (Wells & Ridley, 2005). This simple and practical technique represented a major step forward in the study of intracellular dynamics involved in chemotaxis and haptotaxis, as the Dunn chamber brought together imaging and asymmetric orientation of cells in vitro.

b) Microfluidic-based chemoattractant systems

Despite the advance that the Dunn chamber represented in studying cell migration, it did not enable precise control of concentration gradients. In Dunn

chambers, concentration gradients were established by an approximated diffusion of chemoattractant that was not maintained at constant and precise concentration profiles, nor could diffusion be controlled to establish complex concentration landscapes. This limitation has in the past decade been addressed by photolithographic techniques that enable microfabrication of fluidic networks for precise control of diffusive mixing processes. This approach is broadly called microfluidics, and the application of microfluidics has been pioneered primarily by George Whitesides and coworkers at Harvard University (Sia & Whitesides, 2003; Park & Schuler, 2003; Li et al., 2003).

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CHAPTER TWO:
USING MICROFLUIDIC CHANNEL NETWORKS TO GENERATE GRADIENTS FOR STUDYING CELL MIGRATION

* Daniel S. Rhoads, Sharvari Nadkarni, Loling Song, Camilla Voeltz, Eberhard Bodenschatz and Jun-Lin Guan. 2005. Using Microfluidic Channel Networks to Generate Gradients for Studying Cell Migration. Methods in Molecular Biology 294: 347-357.

I. Abstract

In this chapter, we will discuss a method for the generation of gradients that can be quantitatively used for studying directional cell migration. Microfluidic networks serially split and re-mix small volumes of solutions under laminar flow conditions to generate a series of microchannels of increasing protein concentration. At a juncture of these microchannels, where a single broad channel is formed, a protein concentration gradient can be easily achieved. This method is highly useful because it allows one to control, manipulate, and analyze chemical gradients and cells' chemotactic behavior in a quantitative manner.

II. Introduction

The merger of physics, engineering and biological disciplines into what is currently termed nanobiotechnology has allowed for the development of a number of novel techniques that can be used to study biological activities on very small scales. This area of research has sprung from an outgrowth of the microprocessor industry, which for years has sought to fabricate smaller and smaller structures using photolithography. Photolithography uses epoxy-resins that when illuminated by specific wavelengths of light, cross-link to form insoluble microstructures, to pattern a series of physical features onto a silicon substrate (Microchem Corporation, http://microchem.com/). In patterning microfluidic networks, a negative acting epoxy resin with broadband sensitivity in the near-UV called SU-8 is the standard resist, which is ideal for thick features having high aspect ratios (2-500μm high off the substrate). The raised features formed by the patterned SU-8 then act as a negative mold for rapid prototyping, a process which uses a silicone elastomer, poly(dimethylsiloxane) (PDMS), to form an optically transparent network of microchannels. Bonding

the PDMS to a glass slide then seals these microchannels (Liedberg & Tengvall, 1995; Liedberg et al., 1997).

The microfluidic device is then used to make a biochemical gradient. Generation of the gradient is based upon the controlled diffusive mixing of low Reynolds' number fluids by repeated splitting, mixing, and recombination of fluid streams within the network of microchannels. This gradient can be composed of virtually any biologically relevant molecule, either in solution or deposited on a surface, depending upon the characteristics of the molecule in question. Here, in studying chemotactic cell migration we can generate a soluble gradient of growth factors or chemokines, or in studying haptotaxis, deposit a surface gradient of extracellular matrix (ECM) proteins.

Gradients of these and other classes of molecules play important roles in a variety of biological processes such as morphogenesis, angiogenesis, axon pathfinding, immunological response, and tumor metastasis, to name a few. While other techniques are available for studying cell migration, such as the Boyden chamber or micropipets to release diffusible molecules, few are capable of generating and controlling spatially and temporally well-defined gradients and allowing data analysis in a quantitative manner. These characteristics make migration studies on concentration gradients a useful tool in studying dynamic processes that enable cells to sense and react to chemical changes in their local environment.

A variety of methods have been described for generating concentration gradients of bio-molecules, including diffusion of alkanethiols through porous matrices (Herbert et al., 1997; Terrill et al., 2000), photochemical activation of self-assembled monolayers (SAMs) for peptide coupling (Terrill et al., 2000; Caelen et al., 2000), electrochemical desorption of SAMs (Bradke & Dotti, 1999), depletion of protein inside microfluidic channels by adsorption (Gallardo

et al., 1999), and use of pipettes for forming transient gradients in solution (Weiner et al., 1999; Wilkinson, 1998; Jeon et al., 2000; Dertinger et al., 2001), in addition to the use of pyramidal microfluidic networks (Stroock et al., 2002; Duffy et al., 1998). While each of these can be useful in particular studies, pyramidal networks offer quantifiable gradients that make possible the quantitative study of cellular responses under a variety of spatially and temporally tailored chemical gradients.

III. Materials

Clean-room. Patterns were designed with Symbad pattern editor software, and transferred to the Cornell Nano-scale Facility (CNF) PG3600F optical pattern generator, which patterned our 5-inch mask. Development and etching of the mask were subsequently done in CNF chemical hoods.

Silicon wafers were spin-coated with SU-8 negative photoresist. The patterns were then transferred by soft contact exposure through the mask, using an EVG-620 contact aligner, baked at 95º C. for one hour, and developed in SU-8 developer solvent.

Microfluidic device molding and assembly. Poly(dimethylsiloxane) (PDMS) was provided as the Sylgard® 184 silicone elastomer kit from Dow Corning, Inc., and was used at the standard 10:1 ratio of siloxane base to curing agent. After degassing and baking, PDMS was plasma cleaned, and bonded to glass slides. Gastight® syringes with luer-lock hubs adjoining 24-gauge Teflon tubing, from Hamilton Corp., were attached to the device. A PHD2000 dual syringe pump was used to control syringes and generate microfluidic conditions.

Biological Materials. Fibronectin, bovine serum albumin (BSA), FITC-conjugated dextran, and FITC-conjugation kit were obtained from Sigma-Aldrich. Antibodies were provided by Dr. Jun-Lin Guan.

IV. Design and Development

Microfluidic network design based upon estimated diffusion coefficient for fibronectin. The pyramidal microfluidic network that we modified and fabricated is an array of microchannels arranged in a pyramid-like structure. This design serially splits and recombines two fluids of different concentrations to give a continuous linear concentration gradient, and a useful means of arranging these mixing microchannels in a serpentine pattern, to conserve space and maximize diffusive mixing (Figure 2.1).

Design of the gradient generating microfluidic network was based upon estimates of molecular diffusion rates in conditions of low Reynolds' number and low volume. Previous work (Jeon et al., 2000) demonstrated that the constant and quantifiable characteristics of such microfluidic conditions could be applied to generate highly reproducible micro-scale conditions, which we simplified to the following equation:

$$\Delta Y_M = U \bullet w^2 / D,$$

$$\text{Where } D = \sqrt{(k^3/\pi^3 m)} \bullet [T^{3/2}/(a^2 P)].$$

ΔY_M = Mixing Length, U = Flow rate, w = channel width, D = diffusion constant, T = Temperature (°K), P = Pressure, k = Boltzmann constant, m = mass (molecular, of dissolving substance), a = Stokes' diameter

Mixing lengths were varied in successive levels in the pyramid, since flow velocity decreases, requiring less distance for complete diffusive mixing to occur. In approximating the required mixing lengths, we used a flow rate of 100 μm/s as a base value. For other values, channel width used was 50μm; and the diffusion constant (D) for fibronectin was predicted to be 4.07×10^{-11} m^2/s, from $m = 8.3 \times 10^{-22}$ Kg, $a = 2 \times 10^{-8}$ m, and standard atmospheric values for

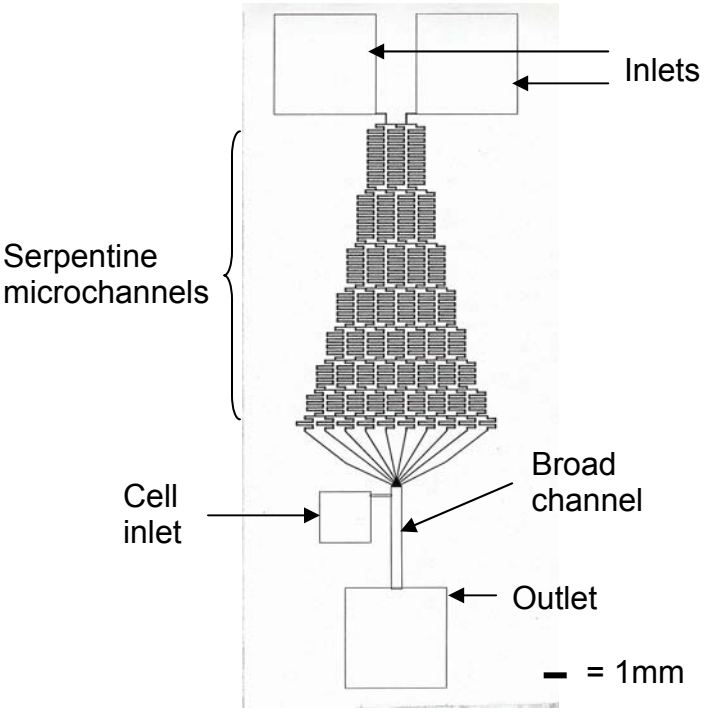

Figure 2.1. CAD printout of a pyramidal microfluidic network design. Important features include the serpentine microchannels that maximize channel length while keeping network size compact, a broad channel for formation of the desired gradient, and inlet/outlet ports. The port to the left of the broad channel may be found useful for introducing cells for migration experiments under many conditions, especially for soluble gradients that require continuous flow during the experiment. Microchannels are 50μm wide. Bar equals 1mm.

T and P. From this, we determined the changing values for flow rate (U) and mixing length (ΔY_M) for each successive layer in the serpentine mixing microchannels (Table 2.1).

Mask and wafer generation. These length values were incorporated into the computer-assisted design (CAD) pattern (Figure 2.1), which was then scanned onto an optical mask using a Cornell Nano-scale Facility (CNF) optical pattern generator. The mask was a glass plate coated with a layer of about 80nm sputtered chromium, coated overtop with a photoresist. After exposure and development, the chromium is removed from the unprotected areas with an acid etch, and an image of the pattern is left in the chromium. The resulting mask was used to limit cross-linking of a negative photoresist (SU-8 100, MicroChem Corp.), which was spin-coated onto silicon wafers to create master molds.

Evaluation of SU-8 and PDMS patterning during microfabrication. As described schematically in Figure 2.2, the generation of wafers patterned with SU-8 photoresist was the next step in developing our microfluidic device. To evaluate the wafers we patterned, SU-8 coating thickness was examined at various spin rates, estimated from MicroChem Corp. specifications, and pattern height was measured by surface profiling (Figure 2.3). We found that pattern height varied by ±5μm for features of about 100μm, when SU-8 100 formulation was spin-coated in the following conditions: 500rpm for 5s, with 100rpm/s acceleration, followed by 3000rpm for 30s, with 300rpm/s acceleration. This was found to be more consistent than using the SU-8 50 formulation, which had a lower viscosity, and worked best when spun at a maximum of about 1450rpm. The SU-8 50 formulation varied by more than twice as much as the 100 formulation for heights of 100-micrometers (data not

Table 2.1. Calculations of diffusive mixing lengths. Based on a desired flow velocity of approximately 100μm per second in the large gradient-forming channel, we determined the fluid volume involved (0.15-microliters/min per syringe). Numbers calculated above are for levels of the network with the indicated number of diffusive mixing microchannels. From this, we calculated the flow velocities at each level of the serpentine mixing network, and the required mixing lengths required for fibronectin mixing to occur by diffusion. This information was used in designing the network pattern shown in Figure 2.1.

Microchannels	Estimated flow rate	Estimated Length for 100% mixing	Length patterned
3	0.50mm/s	20.5mm	19250um
4	0.33mm/s	15.4mm	15450um
5	0.25mm/s	12.3mm	12600um
6	0.20mm/s	10.3mm	10700um
7	0.17mm/s	8.8mm	8800um
8	0.14mm/s	7.7mm	7850um
9	0.11mm/s	6.9mm	6900um
10	0.10mm/s	6.2mm	6000um

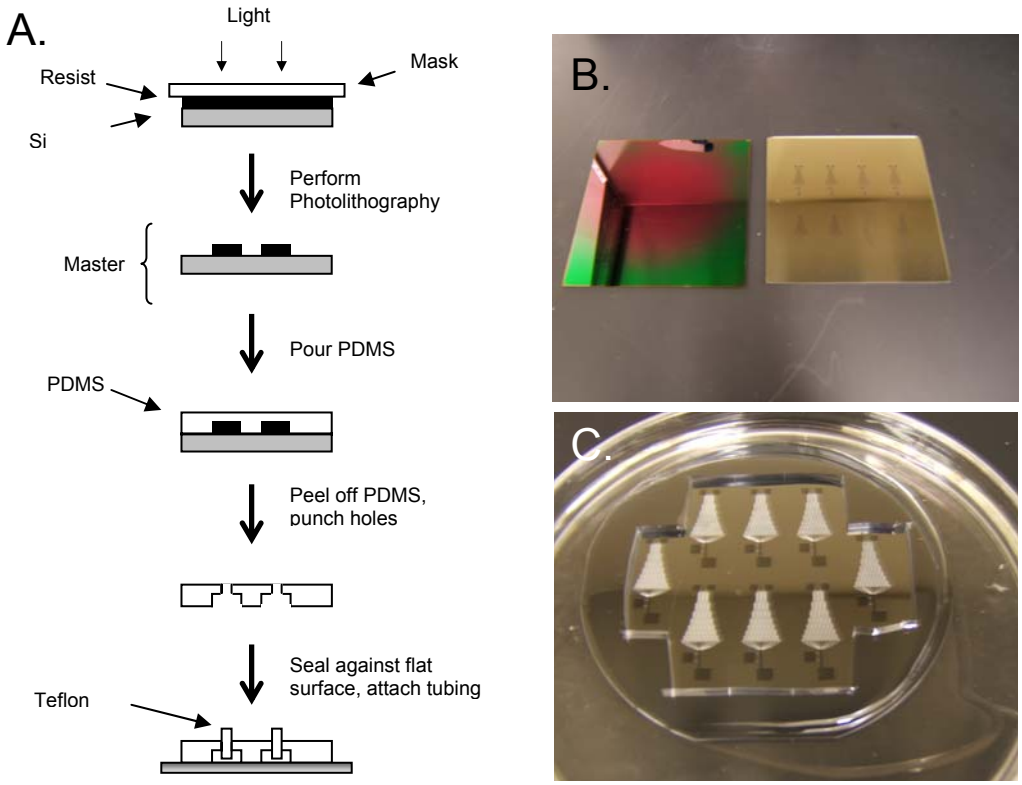

Figure 2.2. : **From mask to microfluidic device.** (A) A chrome mask etched with a microfluidic network pattern is exposed through to cross-link SU-8 resist, transferring the pattern by photolithography. After development, the wafer retains the SU-8 pattern and can function as a master for molding PDMS stamps. PDMS is poured over the pattern and cured, then peeled off, and holes are punched to provide access to fluid ports. The PDMS stamp is then sealed to a flat surface and tubing is attached via punched holes. (B) An unpatterned mask coated with photoresist, and a patterned mask with photoresist removed and the pattern etched into the chrome coating. (C) A patterned wafer and PDMS; the PDMS here has been peeled off and bonded to glass slides.

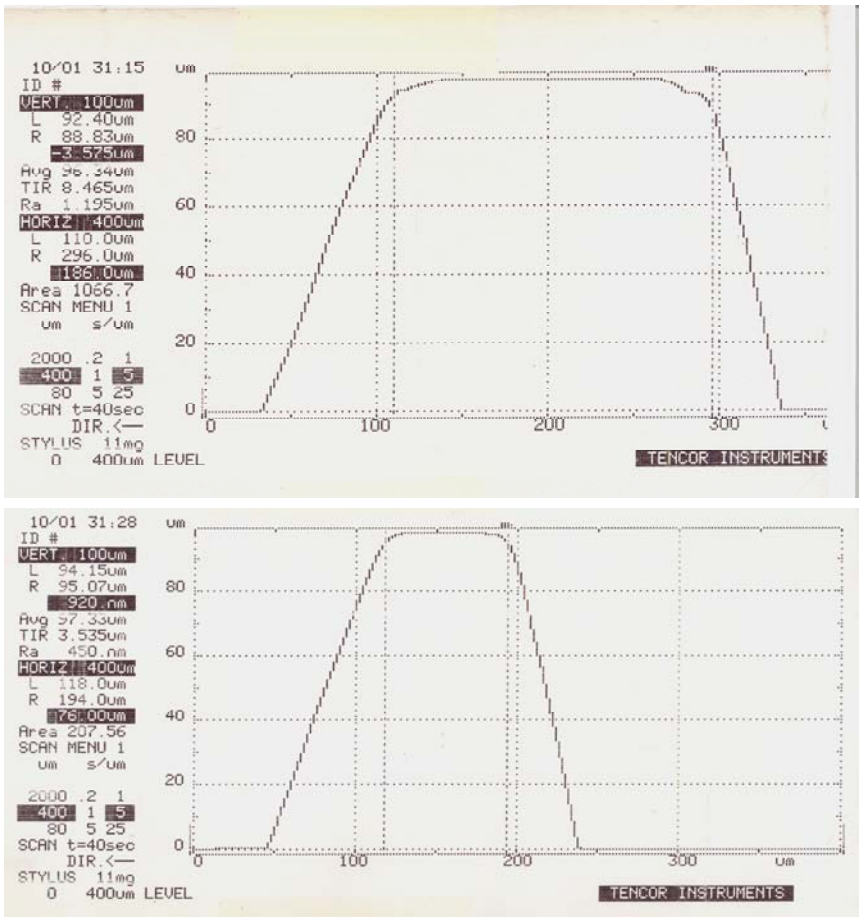

Figure 2.3. SU-8 spinning and wafer surface profiling. Original SU-8-patterned wafers varied both in feature width and height. Variation in width was largely due to underdeveloping un-crosslinked SU-8 with solvent. Height variation was, in these measurements of SU-8 50 formulation, as great as 13-micrometers (13%), but this was later reduced to 5% with the use of the SU-8 100 formulation (data not shown). Units shown are in micrometers.

shown).

Assembling the device. SU-8 molds were subsequently used to cast microchannel patterns into PDMS, a silicone elastomer that is transparent and biologically inert (Figure 2.2). PDMS molding was done according to suppliers' recommendations, using approximately a 10:1 ratio of siloxane base to curing agent, which were mixed, degassed under vacuum pressure for 2 hours, and baked at 60º C. for an additional 2 hours. After the PDMS solidified, we cut the network-molded stamps out, punched inlet and outlet access ports with a 19-gauge stainless steel capillary tube, plasma cleaned the stamp, and bonded it to a glass microscope slide. Plasma cleaning was performed with a coverslip over the lower third of the stamp, so bonding was limited to the serpentine mixing network, and the broad channel containing the deposited gradient could be later removed and used with cultured cells in migration assays. Immediately after bonding to the glass slide, microfluidic devices were stored in distilled water until used. When used, 24-gauge Teflon® tubing was inserted into the inlet ports, connecting them to a syringe pump (Figure 2.4).

Characterization of flow rates and diffusive mixing

Gradient generation was calibrated with fluoroscein isothiocyanate (FITC)-conjugated dextran, which immediately established a consistent fluorescent gradient. Time-course examination of gradient stability was not necessary, as the gradient relied on complete mixing at each level. Thus, the mixing of FITC-dextran was measured via changes in fluorescence at the beginning and end of various mixing levels (Figure 2.5.A-B). Additionally, the merger of microchannels into the final region was imaged (Figure 2.5.C), as well as the linearity of the gradient approximately 100-μm below that (Figure 2.5.D).

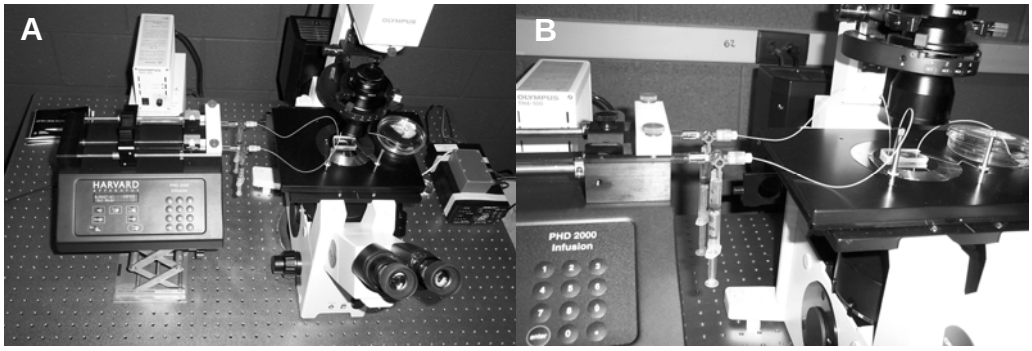

Figure 2.4. Microfluidics experimental setup. Left, view of entire microfluidic setup with syringe pump, connected syringes, and PDMS stamp on microscope (A). Right, closer image of syringes, tubing and PDMS stamp (B).

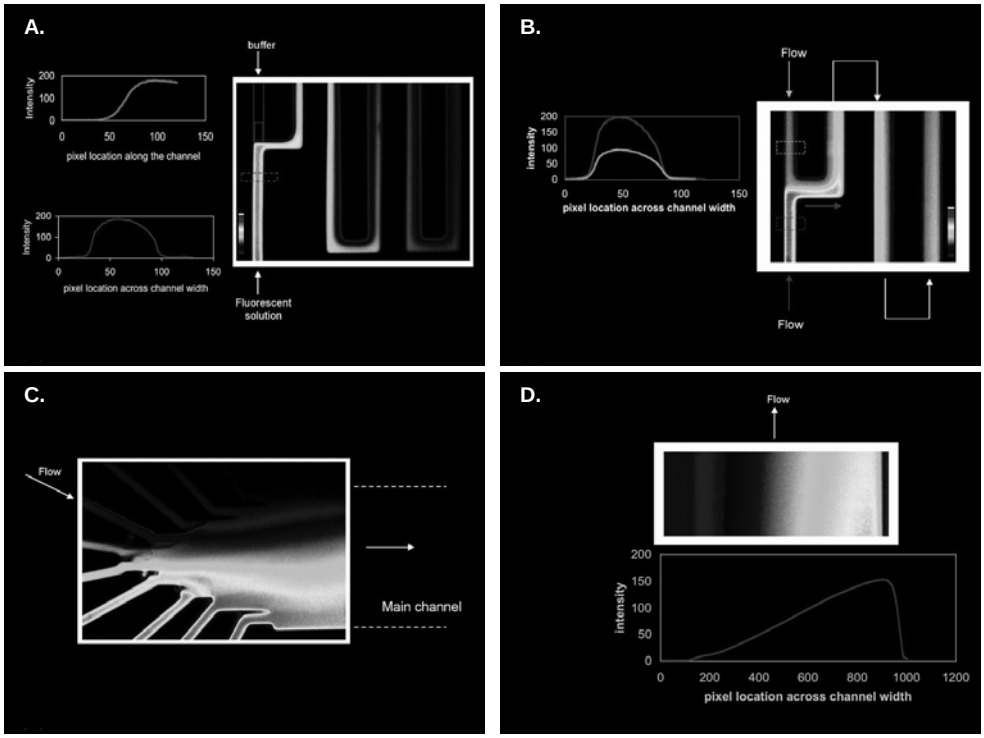

Figure 2.5. Diffusive mixing and gradient generation with a pyramidal microfluidic network. A and B, colorimetric images of diffusive mixing between 0% and 100%, and 25% and 75%, concentrations of Dextran-FITC within a serpentine microchannel, respectively. C and D, colorimetric images of gradient formation as the microchannels rejoin into the broad channel, and an intensity profile analysis of the resulting gradient, respectively. Arrows indicate direction of flow within the networks.

Fibronectin gradient profiling. Generating gradients of fibronectin presented additional challenges beyond that of soluble molecules such as dextran. As deposition of fibronectin is not instantaneous, we had to estimate the rate of protein adsorption onto the surface, the amount of protein required to coat the microchannel surfaces. We also estimated an appropriate concentration of protein to use, in the process of confirming that we could generate linear surface concentration gradients of fibronectin. To address these issues, we obtained and compared two antibodies for immuno-reactivity to fibronectin: the mouse monoclonal antibody Br5.4 and the rabbit polyclonal antibody 62.2. The latter (62.2) produced noticeable signal with little background at 1:400 dilution, whereas Br5.4 failed, and 62.2 was used for further testing (data not shown).

To examine gradient-generating conditions, we generated gradients by introducing the following with syringe pump to the device inlets: 5µg/ml each of bovine serum albumin (BSA) in both; 5µg/ml each of BSA and fibronectin; 25µg/ml BSA and fibronectin; and 25µg/ml fibronectin in both. These were deposited at a high flow rate (500µm/s) for 30min each, and then immunostained with 62.2 antibody (Figure 2.6A-D). While 500µm/s theoretically did not enable complete mixing in the serpentine network, it permitted enough mixing for a gradient to be visualized. More importantly, this experiment suggested that 0.4µg of fibronectin or less (the amount used with 5µg/ml mixture) was an insufficient amount of protein for gradient deposition, but that 1.7µg worked acceptably well. From these results, we determined that 100µm/s, or about 0.075µl/s, was a desirable flow rate. For amount of fibronectin to use, we opted to provide this in excess, to ensure complete coating of microchannel surfaces, so we chose to use 25µg (or 100µg/ml) of both fibronectin and BSA for each experiment.

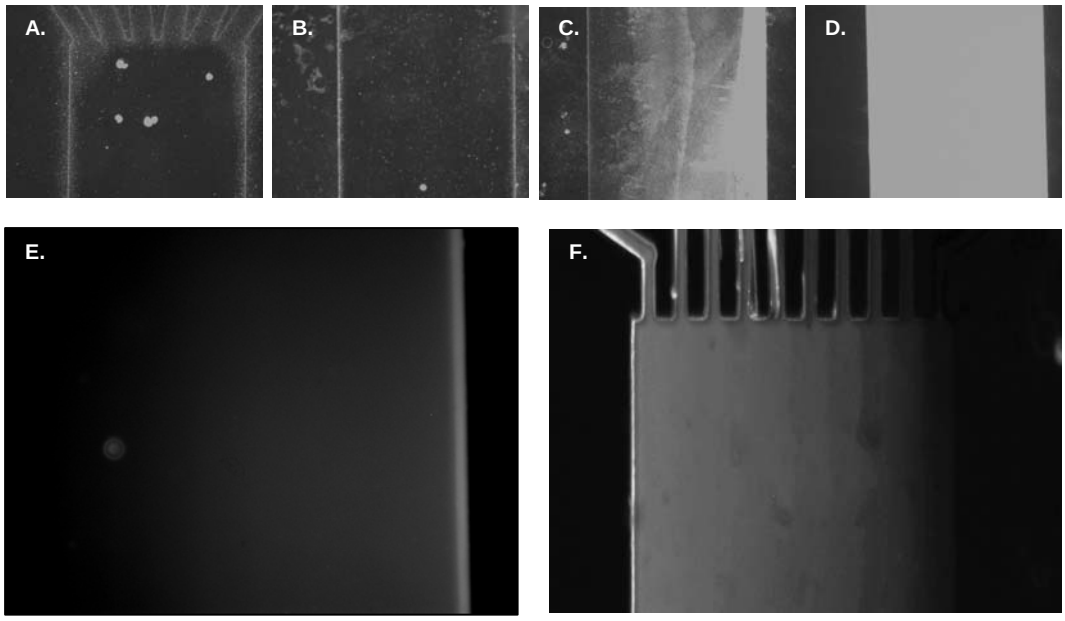

Figure 2.6. Fibronectin gradient imaging. Immunofluorescence of serum albumin negative control (A), 5μg/ml fibronectin gradient (B), 25μg/ml fibronectin gradient (C), and fibronectin positive control coating (D), suggested a baseline fibronectin concentration for use in generating gradients of 25μg/ml or more. Better images were obtained, however, by directly tagging fibronectin with FITC (E), and by directly tagging serum albumin with FITC (F). Channel widths were 750μm.

Visualization of fibronectin gradients with immunostaining was very unreliable, however, and failed to illustrate a constant, reproducible, linear concentration profile. To successfully demonstrate these characteristic of our fibronectin gradients, we conjugated FITC to fibronectin (Figure 2.6E) and BSA (Figure 2.6F) and generated gradients with these. The FITC-Fibronectin gradient was extremely faint, owing to the low level of conjugation that we produced. FITC-BSA, however, produced a much higher conjugate yield, and deposited a constant, reproducible and linear concentration profile.

V. Discussion

Methodologies for imaging migrating cells under quantifiable attractant conditions is of critical interest for the advancement of understanding of biological processes such as metastasis and immune surveillance. In this project, we adapted a microfluidic network concept conceived by George Whitesides and colleagues (Jeon et al., 2000). This concept, consisting of a pyramidal array of microchannels fabricated by soft lithography (Whitesides et al., 2001), uses basic principles of diffusive mixing of minute fluid volumes under laminar conditions (Ismagilov et al., 2000). Because of the channel lengths required for diffusive mixing, we designed the network in a serpentine pattern, to conserve space. An alternative to using serpentine microchannels in a network pattern, which provide enough time for diffusive mixing to occur, is to incorporate Staggered Herringbone Mixers (SHM), which facilitate chaotic mixing in laminar flow conditions (Stroock et al., 2002).

The tendency of fibronectin to adsorb onto silicon-based surfaces, glass versus PDMS in this case, was a major unknown in generating fibronectin gradients with our microfluidic device. We found, quite strikingly, that fibronectin preferentially adsorbed onto PDMS, rather than the glass slide to

which the network-imprinted PDMS stamp was bonded (data not shown). Toworfe and colleagues report similar observations, and suggest that cell function was enhanced more on fibronectin-coated PDMS that was treated with oxygen plasma cleaning, than fibronectin-coated PDMS that was not treated (Toworfe et al., 2004). Similar work has suggested that oxidized PDMS is ideal for culturing NIH-3T3 cells, among others, and that fibronectin-coated PDMS is a suitable cell culture environment for most cell types (Lee et al., 2004). Additionally, the use of the network-imprinted PDMS surface for conducting cell migration experiments was aided by the easier visualization of the gradient boundaries. As a result, separating the PDMS from the glass slides after gradient deposition, and using the PDMS surface to seed and track motile cells, proved to be a convenient system.

Gradient generation for analysis of cell migration, using microfluidic methods such as diffusive mixing in this pyramidal network, is still a novel method. For gradient deposition onto surfaces, for instance, the primary proof-of-concept study done involved laminin gradients for directing axonal processes in neurons (Dertinger et al., 2002). Additionally, the possibility of generating surface gradients of soluble molecules, using biotin/avidin interactions, has been explored (Jiang et al., 2005). More development into the generation of gradients in solution has been performed, focusing primarily on growth factors and chemokines. Examples of such studies include IL-8 for neutrophils, IL-8 and LTB4 for neutrophils, and EGF gradients for MDA-MB-231 breast cancer cells, (Jeon et al., 2002; Lin et al., 2005; Saadi et al., 2006).

These methods offer a handful of opportunities for gained understanding of cell migration. First, the relative importance of various cell attractants and polarity determinants can be quantitatively measured, thereby helping to direct future research emphases. In essence, this application can be seen as a

screening process, to help sort out the roles of the complex assortment of coordinating extracellular signals in the cell's microenvironment. Second, the physical roles of surface density, receptor availability and saturation, cytoskeleton tension forces, and cell spreading and adhesion can be examined. For instance, the biphasic pattern of migration rate to varying concentrations of attractants, as predicted by mathematical models (DiMilla et al., 1991), could be more precisely tested in experimental conditions using linear gradients. And a third opportunity is that of combining such gradient conditions with imaging of intracellular protein dynamics. The localizations and interactions of focal adhesion proteins, among others, are of vital interest to understanding the molecular basis for cell migration in development and disease.

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CHAPTER THREE:
ANALYSIS OF DIRECTIONAL CELL MIGRATION ON DEFINED FIBRONECTIN GRADIENTS: ROLE OF INTRACELLULAR SIGNALING MOLECULES

* Daniel S. Rhoads and Jun-Lin Guan. 2007. Analysis of Directional Cell Migration on Defined Fibronectin Gradients: Role of Intracellular Signaling Molecules.

I. Abstract

Directional cell migration on ECM plays important roles in embryonic development and in several processes in adult organisms. To study the mechanisms and signaling pathways involved in the regulation of directional cell migration, we created defined FN gradients using microfluidic systems. We found that fibroblasts exhibited haptotaxis towards higher FN concentrations on the gradient. Furthermore, the net movements in the direction of FN gradients correlated with the increase in the slope of the gradient although the overall rate of migration was not correlated. Consistent with previous observations on the uniformly coated surface, local higher FN concentrations led to reduced migration rates due to increased spreading. Upon transfection with N-WASP or activated Cdc42, but not FAK or Grb7, the cells showed increased directional migration. However, transfection with FAK, but not the other signaling molecules, led to an increase in the persistence of directional cell migration, which is dependent on the slope of the gradient. Together, these studies reveal some novel properties of directional cell migration on defined FN gradients and suggests a role for FAK signaling and N-WASP and Cdc42 in the differential regulation of the persistence and rate of directional cell migration.

II. Introduction

Cell migration is a complex process essential for embryonic development and life. For certain cell types, migration can become polarized in response to an extracellular stimulus causing cells to travel in a unidirectional manner. For example, epithelial cells migrate in wound healing processes, vascular endothelial cells migrate to form new capillaries during angiogenesis, leukocytes migrate to sites of inflammation and infection, and cell sheets migrate to form tissues and organs during development (Gilbert, 2003; Singer &

Clark, 1999; Moser et al., 2004). Conversely, deregulated cell motility can result in the development of a number of pathologies, including tumor metastasis and angiogenesis, chronic inflammation, and various immune response dysfunctions (Yamaguchi et al., 2005; Wang et al., 2005; Luster et al., 2005).

Cell migration is an integrated multi-step process that involves the coordination of complex biochemical and biomechanical signals to modulate cell morphology by dynamically rearranging cytoskeleton filaments and generate cellular traction (Ridley et al., 2003; Li et al., 2005; Schwartz & Horwitz, 2006). Migration as a cellular process involves protrusion of the leading edge, formation of new adhesions at the front, contraction of the cell, and release of adhesions at the rear, with directional determinants being associated with the mechanisms underlying actin polymerization in cell protrusion and traction generated by contractility of actin filaments. Rho family GTPases are of particular interest, as determinants of how and where actin polymerization and rearrangement occurs – Cdc42 and Rac regulate the Arp2/3 complex through proteins in the Wiskott-Aldrich syndrome protein (WASP) family to promote lamellapodial and filopodial protrusions, while Rho regulates the formation of stress fibers and focal adhesions and actomyosin contractility (Raftopoulou & Hall, 2004).

Besides Rho GTPases, many other intracellular signaling pathways may regulate actin polymerization, by regulating Rho family GTPases or by modulating many of the same downstream effectors. For example, focal adhesion kinase (FAK) and Src family kinases regulate the subcellular localization and activity of N-WASP through tyrosine phosphorylation, which mediates Arp2/3 complex-initiated actin polymerization (Wu et al., 2004), and FAK/Src complexes can also influence Rac and Rho signaling through

phosphorylation of paxillin and p130Cas and their consequent association with Crk (Cary et al., 1998; Schaller & Parsons, 1995). Another pathway influencing directed migration involves the complex formation of FAK with PI3K, as demonstrated by a FAK mutation, D395A, which selectively disrupts its binding to PI3K but not Src. This particular FAK mutant fails to promote cell migration, although it forms a complex with Src and induces p130Cas phosphorylation(Reiske et al., 1999). This role of PI3K in migration is further demonstrated by the blockage of FAK-promoted motility in CHO cells upon the inhibition of PI3K by wortmannin or LY294002.

Although these previous studies clearly establish a role for the signaling pathways in the regulation of cell migration, it is not clear whether some or all of these pathways are involved in the regulation of cellular responses to gradients of ECM proteins in directional cell migration. In contrast to our understanding of signaling pathways involved in chemotactic responses to soluble factors (Devreotes & Janetopoulos, 2003), much less is known about the signal transduction mechanisms in haptotaxis in cell migration on ECM owing to the limited ability to create gradients of immobilized ECM in a quantitative manner (Moissoglu & Schwartz, 2006). Recently George Whitesides and colleagues have developed a method to create quantitative gradients for either soluble factors or ECM proteins using microfluidic techniques (Jeon et al, 2000). By adapting this method, we have recently generated defined FN gradients in a quantitative manner (Rhoads et al., 2005). In this report, we characterized directional migration of fibroblasts on the gradients and explored the role of several signaling pathways in the cellular response to the immobilized FN gradients.

III. Materials and Methods

Cell Culture and Transfection

Chinese Hamster Ovary (CHO) cells have been described previously (Cary et al., 1996), and were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were transfected with PLUS reagent (10 μl), Lipofectamine (10 μl), and 4 μg DNA for 3 hours in 100mm dishes, whereafter the medium was replaced and the cells incubated for 21 hours prior to migration assays.

Generation of FN gradients

Gradients of FN versus BSA in phosphate-buffered saline solutions were created using a PHD 2000 syringe pump (Harvard Apparatus) in a microfluidic device. Microfluidic devices were fabricated in polydimethyl-siloxane (PDMS) at the Nanobiotechnology Center (An NSF-funded Science and Technology Center at Cornell University, Ithaca, New York), as described in chapter 2. Each inlet was connected to a 500 μL GasTight® syringe (Hamilton Co set to a rate of 0.15 μL/min, for approximately 3 hour. After formation of the gradient, the PDMS stamps are removed and immersed in phosphate-saline buffer (PBS) solution until use.

Cell migration assay

PDMS stamps were removed from the slide and placed gradient- side facing upwards in a 60 mm tissue culture dish. The stamp was immersed in PBS until use. Transfected CHO cells were harvested by washing twice with phosphate buffered saline (PBS) and trypsinized in preparation for migration assay. Cells were resuspended in CO_2-independent medium with 0.2% v/v

FBS. For live cell imaging, cells ($10X10^5$) were plated on the fibronectin-coated gradients in 35-mm tissue culture dishes and allowed to attach in an incubation chamber of an inverted Olympus microscope at 37ºC for $\geq$ 1 hour. In some experiments, a fluorescent image was captured to identify transfected cells prior to each migration assay. Time-lapse phase-contrast images were captured at 20 minute intervals using the 64 megapixel shifting pixel camera SPOT-FLEX camera and its SPOT Software program v4.5 (Diagnostic Instruments Inc.). Using ImageJ (National Institute of Health) software with a manual tracking plug-in, pixel coordinates for each cell's centroid in each image frame, and migration paths for each cell for image stacks were determined. For each cell population, mean velocity vectors were calculated, and all error bars shown represent confidence intervals where $p = 0.05$. Persistence values were calculated as a fraction, where the net movement of cells during each 10 hour assay was divided by the total distance of the cells' tracks.

IV. Results

FN gradient induce haptotaxis in CHO cells

To analyze cellular responses to ECM gradients and the role of intracellular signaling pathways, we prepared FN gradients using the microfluidic devices as described in the Materials and Methods. The deposited FN gradients on PDMS were then imaged by immunofluorescent staining using a monoclonal anti-FN antibody followed by FITC-conjugated goat anti-mouse IgG (Figure 3.1, bottom inset). Quantitation of the gradients indicated they were linear with the exception of an approximately 30 μm region near the sides.

Migration characteristics of CHO cells were examined on several different defined FN gradient profiles with the maximal density of deposited

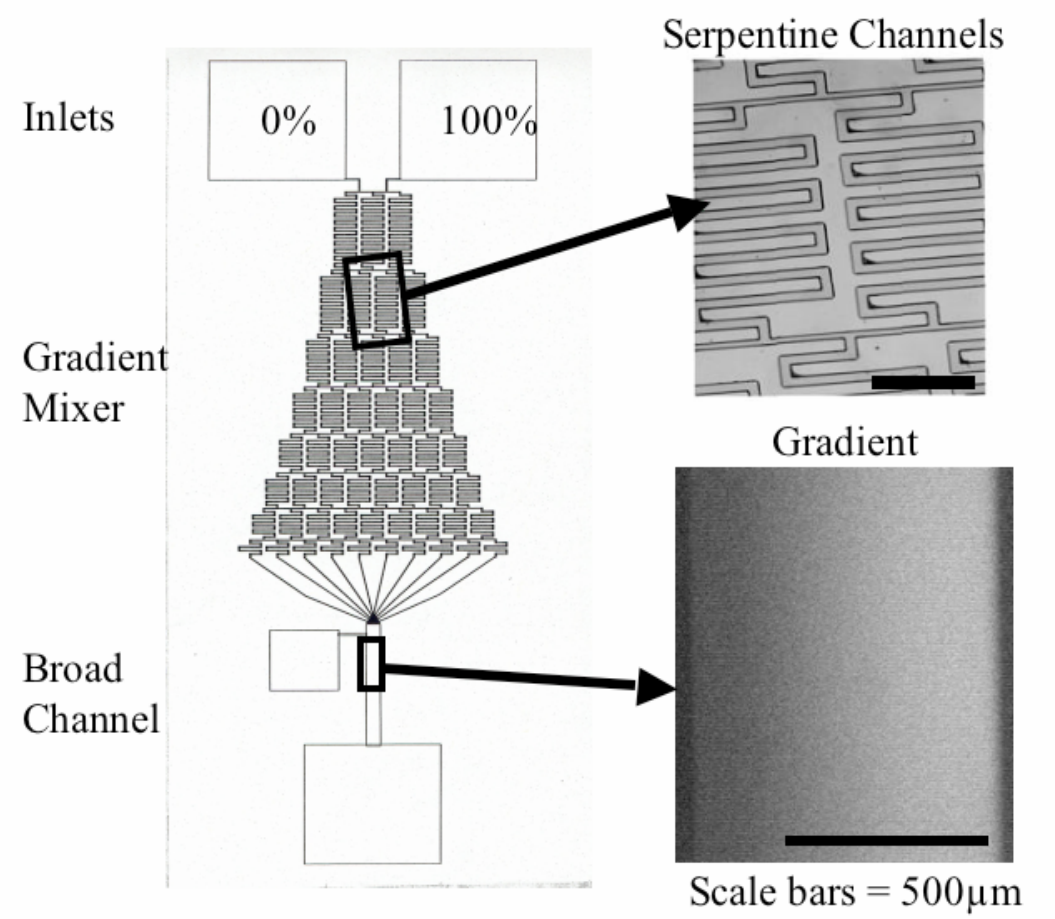

Figure 3.1. Deposition of fibronectin gradients by diffusive mixing. Via two inlets, buffer solutions with and without fibronectin are introduced to a serpentine mixing network. Diffusive mixing takes place in the network, presented as a serpentine pattern to conserve space, creating a series of microchannels with incremental increases in fibronectin concentration. Microchannels are then joined in a broad channel, where flow rate is reduced, blending of adjacent microchannels occurs, and fibronectin is deposited. Top inset: close-up of serpentine microchannels; bottom inset: fluorescent image of FITC-labeled bovine serum albumin (bright) versus unlabeled fibronectin (dark).

fibronectin (Cmax) varied from 25 μg/ml to 100 μg/ml (Figure 3.2A, top panel). CHO cells in a CO_2-independent media were then introduced into the broad channel that had been coated with the FN gradient. Gradients were then placed into a humidified 37°C chamber at atmospheric CO_2 on an inverted Olympus microscope and allowed to adhere for 1 hour. Time-lapse phase contrast images were then captured at 20 min intervals for 10 hours. Using ImageJ software with a manual tracking plug-in, pixel coordinates for each cell's centroid in each image frame, and migration paths for each cell for image stacks were determined. Based on the tracks, the mean overall migration rate (V_0) as well as the directional component towards higher FN concentration (Vx) and perpendicular component (Vy) were calculated (Figure 3.2B). We found that the cells on the FN gradients exhibited haptotaxis towards the higher FN concentration (i.e. $Vx > 0$) whereas the net movement on the perpendicular direction (i.e. Vy) was close to 0 (Figure 3.2C). As expected, cells plated on the control, uniformly coated surfaces (designated as 50 and 100, see Figure 3.2A) did not show any directional preference. Furthermore, we found that cells on steeper gradients showed increased Vx than those on shallower gradients whereas no significant difference for Vy was observed under the various conditions. Surprisingly, we did not find any direct correlation between the slope of the FN gradients and the overall rate of cell locomotion (Figure 3.2D). Cells on the 0-50 gradient showed a greater rate of migration than those on the 0-25 gradient. However, cells on the 0-100 gradient migrated slower than those on the 0-50 gradient. These results suggested that FN gradients could induce haptotaxis of CHO cells, although it did not affect the overall rate of cell migration.

To determine whether the increased directional migration (i.e Vx) on the 0-100 gradient was due to the increased slope of the gradient or the greater

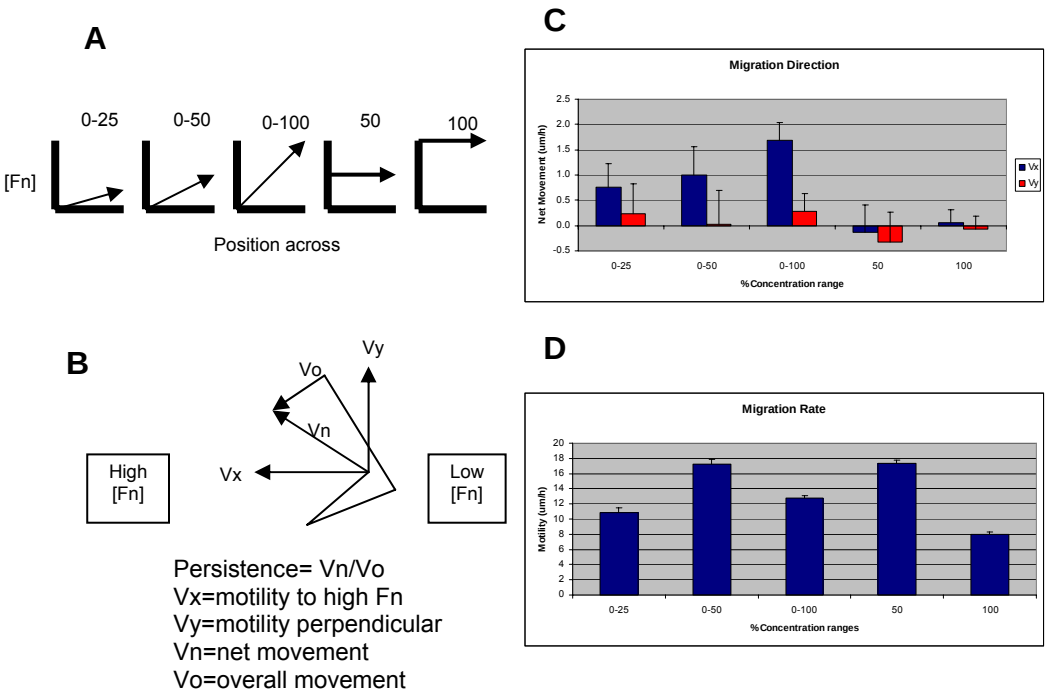

Figure 3.2. Characterization of CHO cell migration on varying fibronectin gradients. Conceptual representations of gradient conditions as fibronectin concentration versus position across the *x* axis (A), and explanation of the parameters examined in haptotactic cells (B). CHO cells on fibronectin gradients displayed increasing directional migration as Cmax was raised (C), which was independent of motility in general (D). Error bars represent confidence intervals of $p = 0.05$; $n >$ 200 cell tracks each.

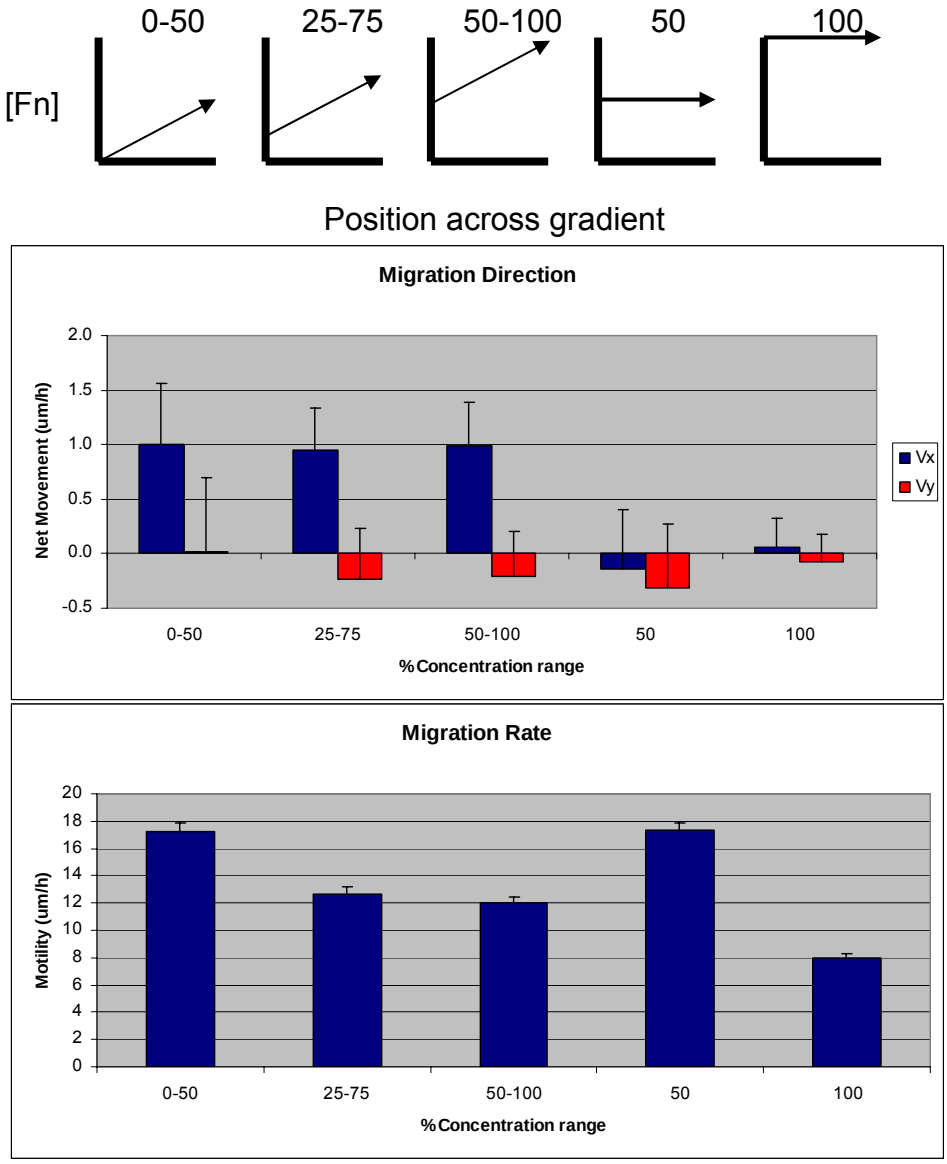

Figure 3.3. Migration rate decreases at higher fibronectin concentrations, but directed migration is unaffected. CHO cells exhibited similar rates of directed movement across conditions of varying fibronectin concentration, but similar gradient steepness (top graph), but motility decreased at higher concentration ranges of fibronectin. Error bars represent confidence intervals of $p = 0.05$; $n >$ 150 cell tracks each.

concentration of FN throughout the gradient compared to 0-25 and 0-50 gradients, we also examined the effects of varying the range of fibronectin concentration with a constant slope of FN gradient. We found similar Vx for cells plated on 0-50, 25-75 and 50-100 gradients (Figures 3.3A and 3.3B), suggesting that the slope of the gradients, but not the concentration of FN, play a more critical role in the induction of haptotaxis on FN gradients. We also observed a slight decrease in the overall rate of migration for cells on 25-75 and 50-100 gradients than those on 0-50 gradient (Figure 3.3C), which is consistent with the earlier observation that the overall migration of cells tends to decrease in high FN concentrations.

High Fibronectin Concentration Promotes Spreading and Reduces Motility

To further explore the dependency of cell migration on FN concentration, we examined the migration of cells across the broad channel (thus with a different local FN concentration) within a defined gradient by plotting the rate of individual cells as a function of their location in the channel. Figure 3.4A shows a gradual decrease of the migration rate with the increase in FN concentration, except for the cells at the very edge of the low FN concentration. Furthermore, this decrease is reflected in the corresponding decrease of Vx (Figure 3.4B) whereas Vy shows no difference across the broad channel. Interestingly, we observed an increase in cell spreading at higher fibronectin concentrations (Figure 3.4D). Together, these results suggest that strong adhesion on the higher FN concentration may be responsible for the reduced migration of these cells.

Cdc42 and N-WASP promoted motility, but FAK promoted directional persistence

A variety of cell signaling molecules and pathways are known to regulate cell migration (Ridley et al., 2003; Vicente-Manzanares et al., 2005),

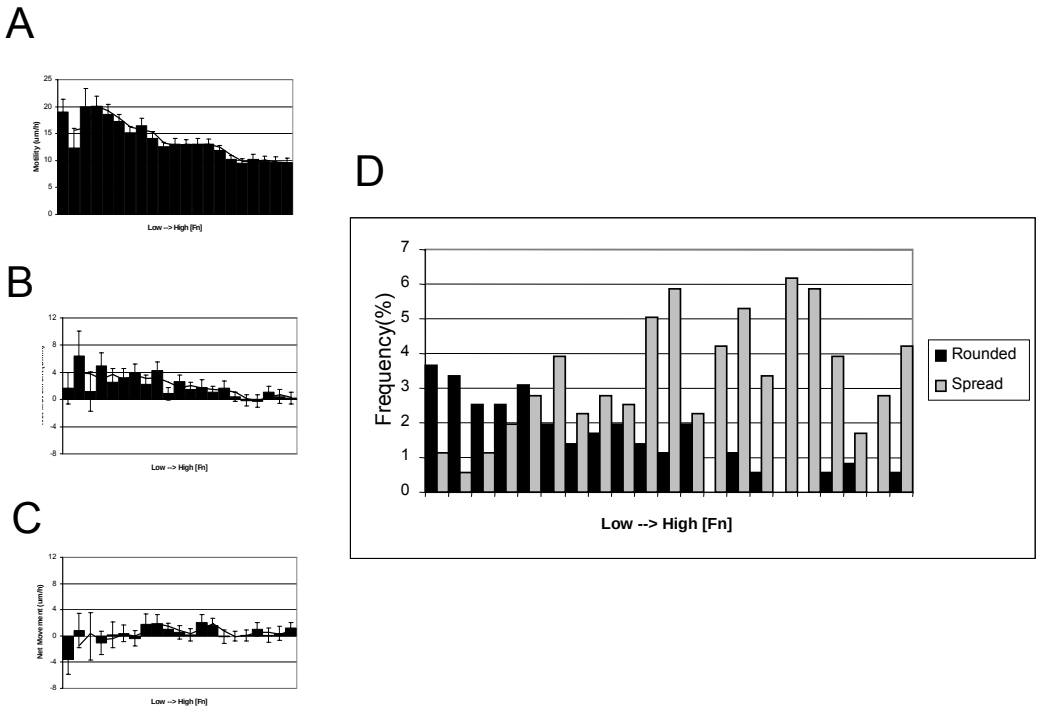

Figure 3.4. Greater fibronectin concentrations correlate with increased cell spreading and reduced motility. CHO cells migrated more slowly (A) and were more spread (D) at higher concentrations. In (D), black represents rounded cells, and grey represents cells scored as spread. Additionally, CHO cells exhibited reduced directed motility on at higher concentrations (B), while non-directional motility fluctuated about zero (C). All graphs are shown as increasing fibronectin concentration from left to right, and error bars represent confidence intervals with p = 0.5; n = 253 cell tracks.

although less is understood about how these pathways may regulate cellular responses to immobilized ECM gradients. Therefore, we investigated the effects of some of the signaling molecules that regulate actin polymerization and focal adhesion dynamics, on the behavior of cell migration on FN gradients. CHO cells were transiently transfected with expression vectors encoding FAK, N-WASP, constitutively activated Cdc42, or Grb7 along with a plasmid encoding GFP. The positively transfected cells were identified by fluorescence microscopy at the beginning of the experiments. Figure 3.5A shows that cells expressing N-WASP and Cdc42, but not FAK or Grb7, showed an increased Vx compared with control expressing GFP alone. These results suggested that Cdc42 and N-WASP, both regulators of actin polymerization important for filopodia protrusion in the leading edge of the migrating cells, play a crucial role in haptotaxis on FN gradients.

FAK has been suggested to regulate the persistency of cell migration on the uniformly coated ECM (Gu et al., 1999), we therefore examined whether FAK could also play a role on the directional migration on FN gradients. We found that FAK increased the persistence of CHO cells on FN gradient whereas N-WASP, Cdc42 or Grb7 did not (Figure 3.5B). Surprisingly, however, the ability of FAK to promote persistence of cell migration was not observed in a shallower (0-25) gradient (Figure 3.5C). These results suggested that FAK could contribute to the persistence of cell migration in FN gradients, which may require certain threshold of the slope of the gradient.

FAK localization not polarized on fibronectin gradients

The potential role of FAK in haptotaxis and directional persistence prompted us to examine its localization on fibronectin gradients, to determine whether gradients induced a polarized recruitment to nascent focal contacts. We found no such polarization in NIH-3T3 cells (Figure 3.6), a finding

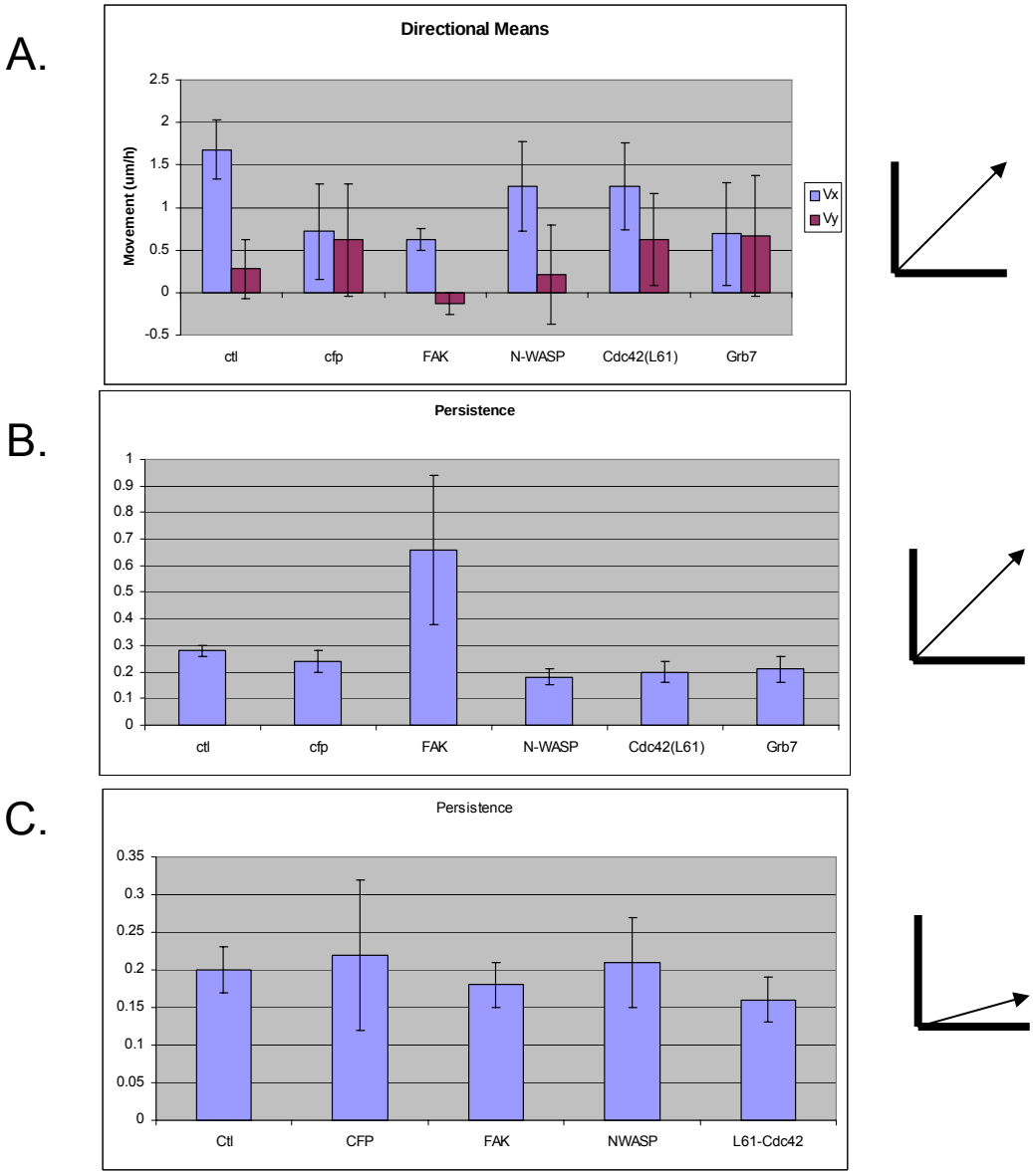

Figure 3.5. FAK, but not Cdc42 or N-WASP, promotes directional persistence. N-WASP- and constitutively active Cdc42-transfected CHO cells exhibited increased directed motility (A), while FAK-transfected cells were significantly more persistent on 0-100 fibronectin gradients (B). This FAK-promoted persistence was not observed on 0-25 gradients (C), suggesting insufficient fibronectin-binding abolishes the haptotactic responses. Error bars represent a confidence interval where $p = 0.05$; at least 20 cell tracks counted for each value.

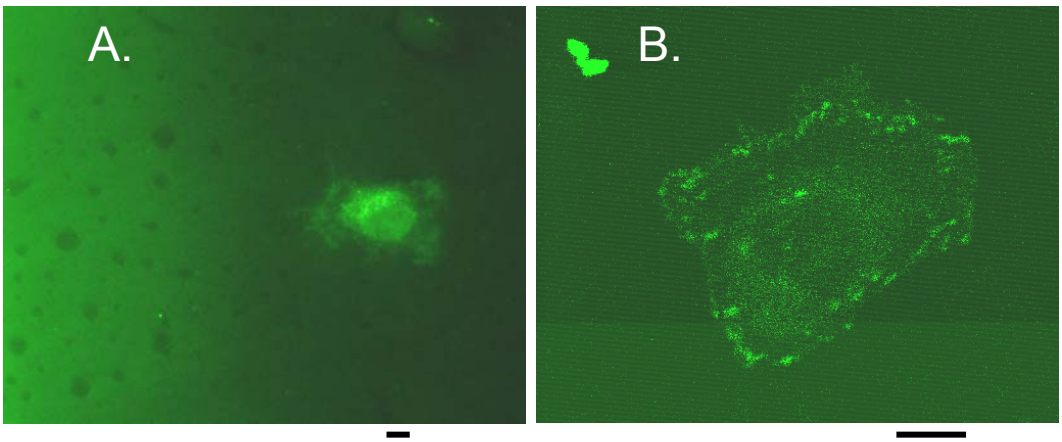

Figure 3.6. FAK localization in haptotactic fibroblasts. NIH-3T3 fibroblasts expressing GFP-FAK on (A) an immunostained fibronectin gradient on PDMS, and (B) uniform fibronectin-coated polystyrene. In both, localized fluorescence is unpolarized. Scale bars are 5um.

consistent with similar experiments conducted by Gu and colleagues (1999). This suggests that the mechanism of FAK-promoted directed migration is more subtle than localization.

V. Discussion

One of the most important aspects of cell migration research is to understand how the cells sense and respond to gradients of chemoattractants and immobilized ECM cues (Moissoglu & Schwartz, 2006). Despite the enormous interest and recent progress in understanding the mechanisms of cell migration, our knowledge about directional cell migration on ECM gradients is limited due to a lack of adequate methods to generate such gradients. We have previously used the Boyden chamber method, where FN gradients were set up via differential coating of the two sides of porous membranes, to study properties by persistence in directional cell migration. Others have used video microscopy to show that FAK plays a role in the persistence of cell migration on uniformly coated ECM surfaces (Gu et al., 1999). It is not clear, however, whether the same signaling pathways are also important for the persistence of cell migration on ECM gradients. In this study, we fabricated and characterized microfluidic devices that allow deposition of defined FN gradients to allow directional cell migration and direct microscopic investigation of the migrating cells based on the pioneering work by Whiteside and colleagues (Jeon et al., 2000). Using such defined FN gradients, we showed haptotaxis of fibroblasts towards higher FN concentrations with a correlation between the directional movement and the steepness of the gradients. However, the overall rate of cell migration was not proportional to the gradients, and indeed it is reduced by the higher local FN concentration as a result of increased cell spreading. While the former is a new and somewhat surprising observation, the latter finding is consistent with the

previously suggested bell shape curve between adhesion strength and migration rate based on studies of uniformly coated surfaces (Palecek et al., 1999, Felsenfeld et al., 1996; Gupton & Waterman-Storer, 2006).

A variety of signaling molecules and pathways have been shown to regulate cell migration, although their roles in the various aspects of cell migration, in particular on directional migration on ECM gradients, are not known. Interestingly, we found that while Cdc42 and N-WASP enhanced directional movement of cells, FAK promoted persistence of cell migration on the gradients. These results re-enforce the idea that different signaling pathways may preferentially affect different aspects of cell migration. Stimulation of actin polymerization by Cdc42 and N-WASP in the leading edge of the cells may be responsible for the directional migration. The possible mechanism by which FAK may regulate persistence of migration is less clear. Gu et al reported that FAK and its downstream target p130cas regulated persistence of cell migration on uniformly coated surfaces (Gu et al., 1999). Thus phosphorylation of p130cas by the FAK/Src complex may also play a role in the persistence in directional cell migration on FN gradient. However, we found that the impact of FAK on the persistence of cell migration on FN gradients was diminished in shallow gradient whereas it is more pronounced on steeper gradient. Therefore, different FAK downstream pathways may be involved in the persistence in directional migration on gradients vs the persistence in random and non-directional migration described in Gu et al. (1999). Our preliminary analysis of FAK mutants suggested that disruption of FAK interaction with PI3K, while retaining its interaction with Src, could abolish the ability of FAK in promoting persistence of migration on the steep gradient (data not shown). These results raised the possibility that while FAK/Src pathway is important for the regulation of random cell migration, the PI3K pathway downstream of FAK may play a

more important role in the promotion of directional persistence. Further analysis will be directed to evaluate these possibilities. Because of the compatibility of the defined FN gradient system with imaging, it is also possible to examine the dynamic localization of the signaling molecules using GFP fusion proteins in migrating cells. These data will complement the studies of using FAK mutants to understand the role of potential differential FAK signaling pathways in the regulation of directional cell migration.

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CHAPTER FOUR:
CONCLUSIONS AND PROSPECTS

I. Conclusions and Prospects

With the tremendous advancements in molecular biology in recent decades, a dynamic model involving the spatial integration of cytoskeleton-related proteins has emerged. Mechanisms driving actin polymerization, supramolecular plaque formation, and generation of traction are all required in cell migration, and all impact each other (Lauffenburger & Horwitz, 1996). Underscoring this model are large volumes of data on the impact of molecules regulating the cytoskeleton (Rafelski & Theriot, 2004), the regulation and turnover of adhesion foci (Webb et al., 2003), and the mechanics of generating picoNewton-scale forces (Li et al., 2005) in motile cells. And, as this model for cell migration as an integrated process has developed, a role for focal adhesion kinase (FAK) has taken on a central role (Moissolgu & Schwartz, 2006; Mitra et al., 2005). FAK is a highly interactive protein, influencing signal transduction coming from a wide range of cell surface receptors, and modulating many cell behaviors. From a biochemical standpoint, therefore, FAK function is both critically important, and yet dependent on a complex network of other signaling modules. And from a mechanical standpoint, FAK appears to mediate the turnover of focal contacts. How to elucidate the molecular basis for FAK function in such an interdependent system then?

In this dissertation, I have described the fabrication of a modified microfluidic device for the purposes of studying haptotactic migration in a simple cell tracking assay (Chapter 2). This device affords numerous advantages for studying migration over previous methods such as the Boyden chamber assay, most notably real-time imaging of motile cells and quantification of cell responses to varying concentration gradient conditions. Using this device, I have provided evidence that FAK overexpression alone is capable of enhancing persistent migration in a direction determined by a cell's

extracellular environment (Chapter 3). FAK did not, however, increase the migration rate of cells in the presence of ECM gradients, suggesting that FAK activity may act to spatially regulate proteins involved in cell polarity. Rho GTPases and PI3K, for instance, operate downstream of FAK signaling, and may in turn positively regulate FAK activity at the leading edge. FAK activity is also influenced by integrin receptor clustering, which occurs at the leading edge of motile cells. It remains unclear how, but it is conceivable that FAK may promote the preference for integrin-ECM ligation at the leading edge. More broadly, however, the suggestion that FAK promotes persistence corresponds with its diverse functions in regulation of the cytoskeleton, adhesions, and contractility. Perhaps by acting at the crossroads of multiple signaling pathways governing such behaviors, FAK is able to upregulate the entire multi-step process of migration, and maintain the balance between each aspect of cell migration.

Suggested mechanisms that might facilitate FAK promotion of migration have included phosphorylation of the Crk-associated substrate (p130cas) and paxillin by the FAK/Src complex (Gu et al., 1999; Webb et al., 2004). Additionally, FAK interaction with PI3K has also been shown to be critical for cell migration (Reiske et al., 1999). To distinguish between these possibilities, we analyzed cells overexpressing FAK mutants, to selectively disrupt SH2 domain-interactions at the phosphotyrosine 397 residue of FAK. These results suggested that while FAK/Src signaling may be necessary for chemokinetic cell migration, FAK/PI3K signaling may be more specifically required for maintaining directed migration. In this model, cooperation with Src may mediate some of the mechanical aspects of focal contact structure, while PI3K-generated phosatidylinositol second messengers may spatially coordinate other processes throughout the cell.

We therefore have proposed that FAK influences cell migration through multiple signaling pathways having distinct functions in focal contact and cell processes, and that among these pathways, PI3K specifically mediates the directional response. Downstream effectors of FAK/PI3K signaling are unclear, but studies point to recruitment of pleckstrin homology (PH) domain-containing proteins, which in turn spatially regulate the GTP/GDP exchange of Rho GTPases and the direction selection of lamellipodia protrusion (Funamoto et al., 2002). Further characterization of such a signaling cascade would strengthen our model, as well as identify potential pharmacological therapies to treat pathologies such as cancer metastasis.

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