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 still survive beyond gastrulation however, and instead exhibit signs that while the mesoderm still 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 â1 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 á and â 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 â3 integrin tail at the NPXY motif via its phosphotyrosine-binding (PTB) domain, and activates integrin receptors by separating the á and â 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 á 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 á4) (Liu et al., 1999).

Significantly greater interest has been focused on protein interactions with the â subunit cytoplasmic tails, however. Cytoskeletal proteins such as talin, á-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 â 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), á-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

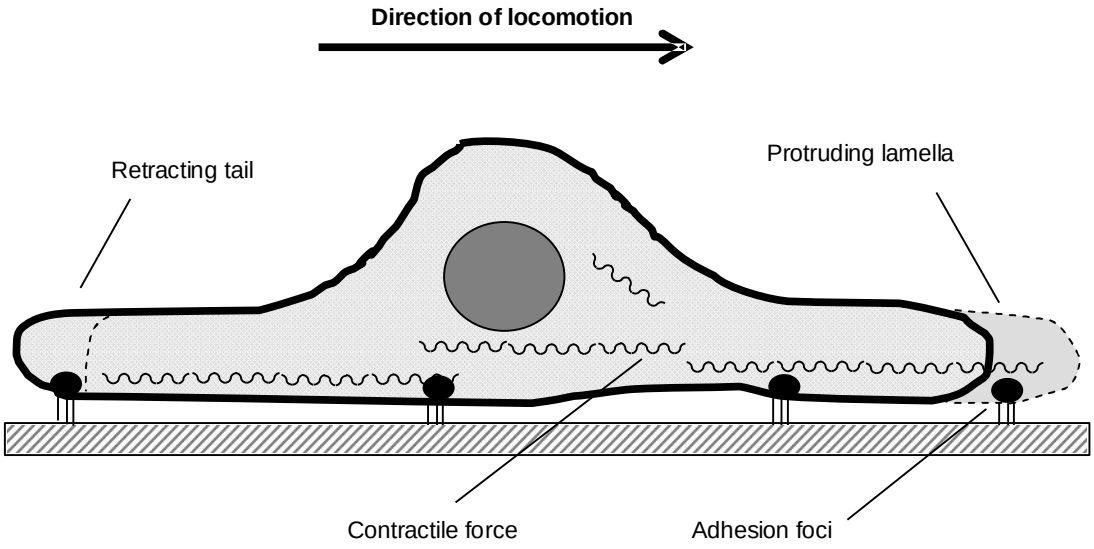


Figure 1.1. Cell migration is an integrated multi-step process. Cell migration involves the spatial coordination of four interrelated processes: Lamellipodial protrusions at the front of motile cells extend the leading edge; adhesion receptors form stable attachments to the extracellular matrix (ECM);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

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 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 á-actinin were also shown to be important in focal complex formation, but GFP-conjugating studies implicated a sequential

recruitment of paxillin and á-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,

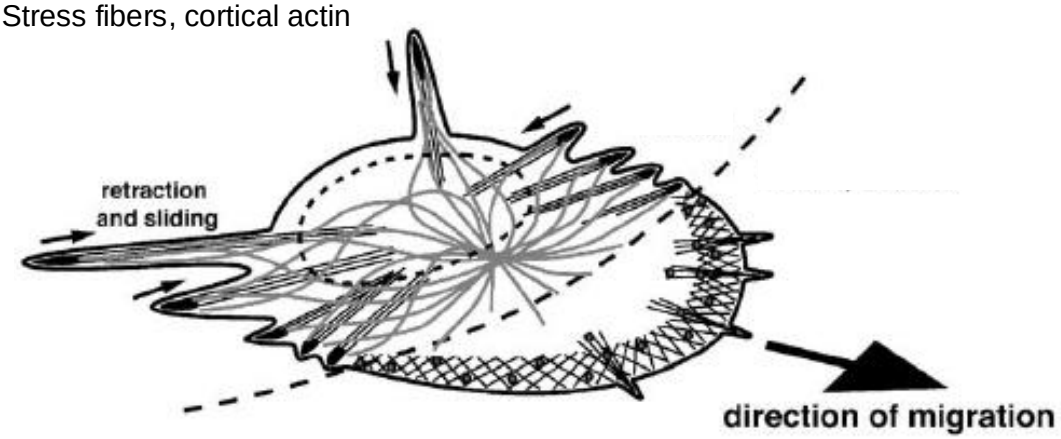
nor are the mechanisms by which Rho is involved in this transition. The dynamics of Rac and Rho will be discussed at greater length in the next section.

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).

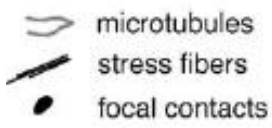
Coupling adhesion, protustion and actin organization is the myosin II-mediated contraction of the actin cytoskeleton (Schwartz & Horwitz, 2006). This model incorporates a regulated coupling of actomyosin contractility and functionalized adhesion foci, which both generates force sufficient for locomotion 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

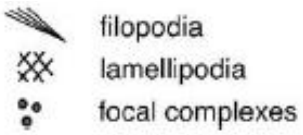
Figure 1.2. Focal adhesions and the actin cytoskeleton perform distinct functions in the front and back of motile cells. In the front, active actin polymerization and rapid turnover of adhesion foci at the base of lamellipodial extend the cell in the direction of locomotion, while at the rear, adhesions 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)

Cell Body
Rho, PTEN, Myosin II
Large, stable adhesions
Stress fibers, cortical actin



Cell Front
Rac, Cdc42, PI3K
Rapid adhesion turnover
Lamellipodia, filopodia





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 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 GTPãS-bound Rac1 and Cdc42, and PI3K activity is enhanced by the GTPãS-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,

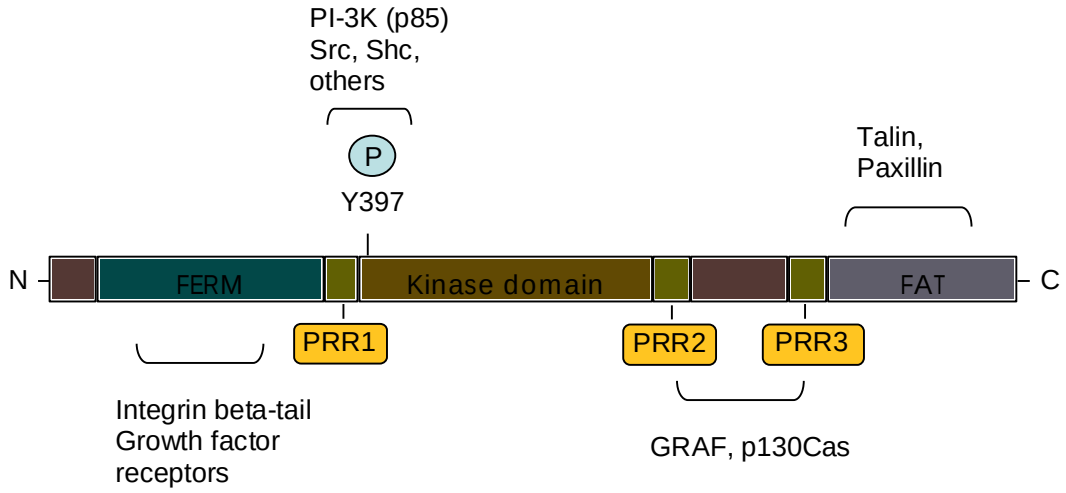
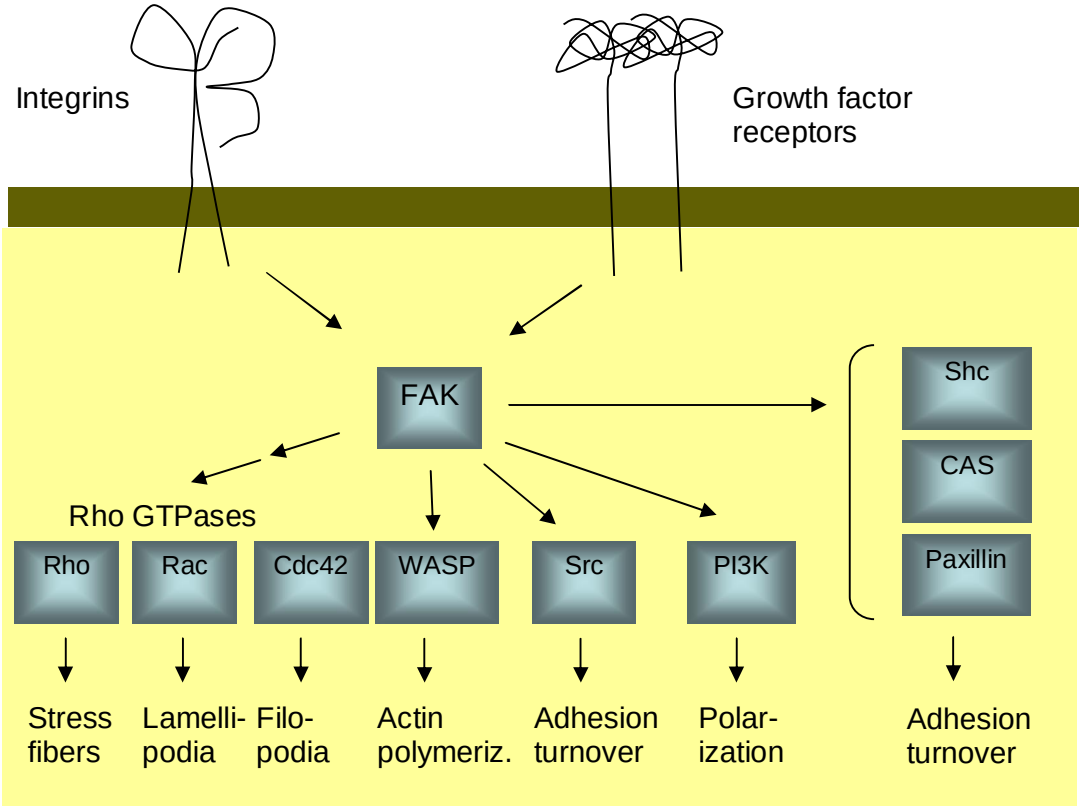


Figure 1.3. Focal Adhesion Kinase (FAK) domains and interactions. FAK contains an N-terminal FERM (protein 4.1, ezrin, radixin, moesin) homology domain, a central kinase domain, three proline-rich regions (PRR), and a C-terminal focal adhesion targeting (FAT) domain. FAT has been shown to be necessary 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.

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, 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.

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.



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 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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