5. MOLECULAR ASPECTS OF CELL MIGRATION

5.1. The beginning of movement: filopodia, lamellipodia, ruffles

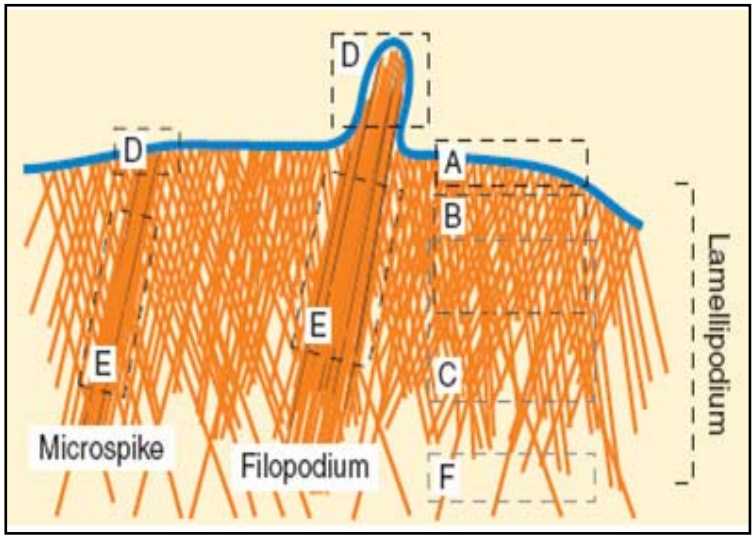
Migration is an essential activity of invading cells. For most authors, the difference between invasion and migration lays in the barriers that cells need to cross to go from one site to another. To simplify, invasion equals migration through an obstructive matrix. Such matrix can be the natural one or a more or less complicated mimic of it. In assays for chemotaxis or chemoinvasion, the direction of migration or invasion is guided by a soluble chemical gradient. Migration is not restricted to invasive cancer cells as it occurs also in normal tissues, such as the intestinal mucosa, and during wound healing. This normal migration is mimicked in culture on solid substrate by wounding of monolayers or by growing cell populations inside collagen type I, where branching morphogenesis occurs. Individual cell motile phenotypes on solid tissue culture substrate are described in terms of stress fibre assembly or membrane ruffling with formation of lamellipodia and fiopodia [26].

The process of cell scattering recapitulates the phenotypical and molecular changes occurring in epithelial cells that undergo epithelial-to-mesenchymal transition (EMT), which prelude to invasion. Cell scatter can be divided into three phases, namely cell spreading, cell–cell dissociation, and cell migration. Therefore, in order for epithelial cells to “scatter”, the attenuation or dissolution of cell-cell adhesions is required. Under normal conditions, the assembly and maintenance of intercellular junctions is tightly regulated. Disassembly of these junctions occurs during certain physiologic and pathologic conditions such as normal development and tumor cell invasion/metastasis. HGF is a potent stimulator of cell scattering, being able to induce cell dissociation and mutual repulsion [24].

To date, the complexity of the molecular machinery that coordinates cell migration both in physiological and pathological condition has only partially been discovered, although several molecules are now well-established key players in the migratory signaling cascade.

Lamellipodia is defined as a thin layer of cytoplasm (~0.2 μm thick) that protrudes at the front of spreading and migrating cells; when lamellipodia curl upwards, they are referred to as “ruffles”. At a molecular level, lamellipodia contain

concentrated arrays of polar actin filaments, that drive the protrusion by actin polymerization.

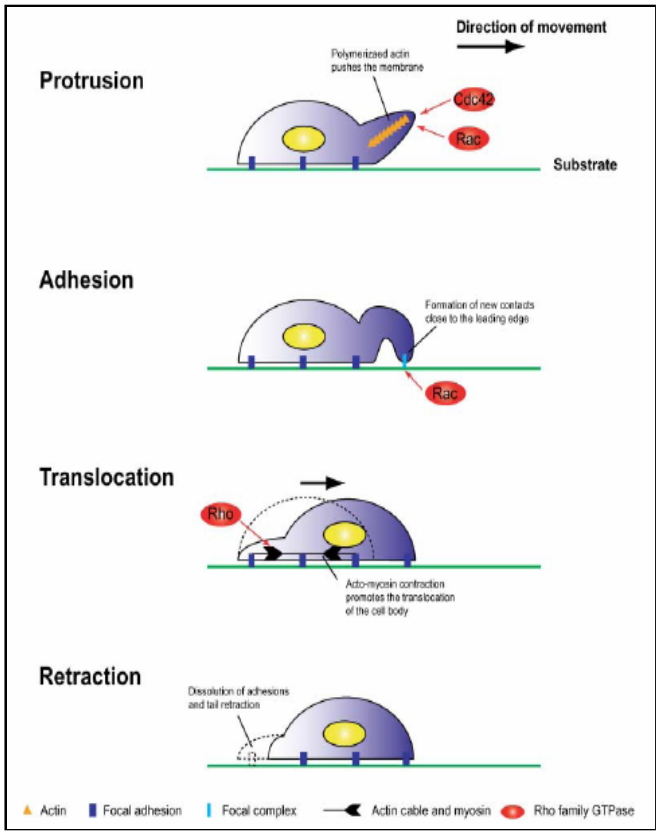


<u>Actin polymerization dirives lamellipodium, microspikes and filipodium formation</u>. From Small *et al.*, TICB 2002 (Ref. 26).

Alongside their protrusive activity, lamellipodia are involved also in the development of adhesions to the substrate and, as ruffles, serve in macropinocytosis and phagocytosis. They must therefore recruit all the components required for these functions. Also, adhesion itself entails reorganization of lamellipodium filaments, leading to the development of different classes of adhesion complexes [26].

Two-dimensional cell movement is induced by four ordered steps: front-membrane protrusion, adhesion of the protrusion to substrate, movement of the cell body, and retraction of the rear part of the cell. These processes are temporally and spatially regulated by Rho family small GTPases, including Rho, Rac, and Cdc42. When cells are stimulated by growth factors, bound GDP is exchanged for GTP. In the GTP-bound state, Rho family small GTPases are active and interact with specific downstream effectors, leading to the translocation and activation of the effectors, and induction of various intracellular responses. Activated Rac and Cdc42 induce reorganization of the actin cytoskeleton at the leading edge. Localized actin polymerization at the leading edge pushes the membrane forward in finger-like structures known as filopodia and in sheet-like structures known as lamellipodia. These structures generate the locomotive force in migrating cells. Unlike Rac and Cdc42, Rho regulates the assembly of contractile acto-myosin filaments. Rho regulates myosin-mediated contractility through downstream effectors, such as

ROCK/Rho kinase. The Rho-mediated acto-myosin contractile force promotes the locomotion of the cell body and the trailing edge. Thus, during cell movement, Rac and Cdc42 stimulate formation of protrusions at the leading edge, and Rho induces retraction at the trailing edge. Rho family proteins interact with a variety of oncogenes such as Dbl, Vav, Lbc, and v-Fms, supporting the putative role of GTP-binding proteins in oncogenic transformation [24,27].



Two dimensional cell movement is the result of four ordered steps. From Yamazaky *et al.*, Cancer Sci. 2005 (Ref. 27).

5.2. From Growth Factors to Rho GTPases: mechanisms for a regulated activation [28]

Rho family small GTPases, beside cycling between the inactive GDP- and the active GTP-bound states, also cycle between the membrane, where active GTPases interact with downstream effectors, and the cytosol, where GTPases interact with GDP dissociation inhibitors (GDIs). Membrane targeting is predominantly mediated by carboxy-terminal sequences that include geranylgeranyl modification and a polybasic motif. Three classes of molecules regulate the activation and/or localization of Rho GTPases: Guanine nucleotide exchange factors (GEFs), which are positive regulators by catalyzing the exchange of GDP for GTP; GTPase activating proteins

(GAPs), which are negative regulators by accelerating the low intrinsic rate of GTP hydrolysis; and GDIs, which act as chaperone proteins that confer solubility in the cytoplasm and inhibit both activation by GEFs and inactivation by intrinsic and GAP-catalyzed GTP hydrolysis. According to the currently accepted model for the activation/targeting cycle of Rho GTPases, inactive Rho GTPases are maintained in the cytosol of quiescent cells bound to RhoGDI. Upon cell stimulation, activation by GEFs occurs coincidently with dissociation from RhoGDI and binding to the membrane. Activated, membrane-bound GTPases then interact with downstream effectors to initiate signaling. Inactivation and termination of signaling involves GAP-stimulated GTP hydrolysis. Integrins promote Rho GTPases membrane targeting by regulating the availability of membrane binding sites so that, in non-adherent cells, active GTPases are present in the cytosol bound to RhoGDI [29,30,31]. Therefore, activation and membrane targeting of Rho GTPases can be separable events. Recently, it has been demonstrated that RhoGDI unexpectedly controls Rac membrane targeting mainly by affecting rates of association.

5.3. Actin Cytoskeleton dynamics: downstream of Rho GTPases

Many recent studies have elucidated the molecular mechanisms underlying Rac- and Cdc42-induced actin reorganization in the formation of filopodia and lamellipodia. In these membrane protrusions, monomeric actin is polymerized into filaments dependent on the Arp2/3 complex. The Arp2/3 complex consists of seven different proteins, including actin-related proteins, which comprise a nucleation core of actin polymerization. Wiskott–Aldrich syndrome protein (WASP) family proteins are key regulators that link extracellular stimuli to actin reorganization and regulate cell migration through induction of membrane protrusions at the leading edge. WASP family proteins link Cdc42- and Rac-dependent signals to filipodium and lamellipodium formation, respectively, by virtue of their ability to activate Arp2/3 complex-mediated *de novo* actin polymerization [27].

WASP proteins interact with phosphoinositides and small GTPases. Phosphoinositides interact with the basic region in WASP and N-WASP. Negatively charged phosphoinositides are thought to associate with the basic region through electrostatic interactions. Next to the basic region, there is a CRIB (Cdc42 and Rac interactive binding) domain. The CRIB region of WASP and N-WASP has been shown

to bind to Cdc42 and other small GTPases related to Cdc42, such as Tc10, RhoT and Chp. Under resting conditions, WASP and N-WASP are folded by an intramolecular interaction between the C-terminal VCA (verprolin-homology, cofilin-homology and acidic) domain and the N-terminal region, which includes the CRIB domain. Folded WASP and N-WASP are inactive because the VCA region is masked, thereby inhibiting access of the Arp2/3 complex to the VCA region. Autoinhibition is released by the competitive binding of other molecules to the CRIB or surrounding regions. Phosphoinositides bind to the basic region and synergize with Cdc42 to induce WASP and N-WASP activation. The binding of SH3 domain-containing proteins to the proline-rich region of WASP and N-WASP activates the Arp2/3 complex, but the precise mechanism of this is not clear. Phosphorylation of WASP and N-WASP by the Src family of tyrosine kinases occurs close to the CRIB region and releases the intramolecular interaction. Importantly, WASP phosphorylation and binding of Cdc42 have a synergistic effect on the activation of the Arp2/3 complex. Therefore, activation of the Arp2/3 complex by WASP and N-WASP is locally optimized by the additive effects of various types of signaling molecules [32].

As opposed to the direct interaction of N-WASP and Cdc42, Scar/WAVE (which transduces Rac-mediated lamellipodium formation via the Arp2/3 complex) cannot bind to Rac directly. A search for the link between Rac and Scar/WAVE led to the identification of the insulin receptor substrate Irsp53. This adaptor protein links activated Rac and Scar/WAVE to induce lamellipodia and is recruited to the tips of both lamellipodia and filopodia [26]. Of the WAVE proteins, WAVE2 is localized to the tips of the lamellipodial protrusions, predominantly in fibroblasts. The binding of basic residues in WAVE2 to PIP_3 mediates its recruitment to the leading edge [27].

Cell shape had long been thought to be determined by the cytoskeleton beneath the cellular membrane. However, this idea could be revised by the discovery of several membrane-deforming proteins that bind to WASP and WAVE proteins. The proteins of the largest population among WASP- and WAVE-binding proteins consist of an N-terminal membrane-binding domain (BAR, EFC or RCB/IMD domain) and a C-terminal SH3 domain. These N-terminal domains bind to the cell membrane and the SH3 domains bind to and activate WASP and WAVE proteins. WASP and EFC-domain-containing proteins probably have been developed for endocytosis or inward vesicle movement. BAR and the EFC domains bind to phosphatidylserine and $P(4,5)P_2$ for

sensing or for the deformation of the membrane, whereas SH3 domains recruit WASP or N-WASP as well as dynamin, leading to the generation of endocytosis vesicles. On the other hand, drastic changes in the shapes of outward protrusions occur during cell movement or cell adhesion. The machinery for endocytosis might have evolved to fill the cell's need to form protrusive structures for cell movement and cell adhesions [32].

Two of the proteins shown to bind to and activate the Arp2/3 complex *in vitro*, cortactin and Abp1, also co-distribute with Arp2/3 across the lamellipodium. Because cortactin can activate Arp2/3 when bound to F-actin and inhibits debranching of *in vitro* Arp2/3-actin complexes, it has been suggested to serve as a stabilizer of the putative actin filament branches in the lamellipodium. As a potential receptor linker, cortactin might couple actin flow to receptors on the surface of the lamellipodium. Abp1 has similar properties to cortactin. Other candidates for actin network stabilization are the classical actin crosslinking proteins filamin and α-actinin. In addition to binding to F-actin, filamin can associate with transmembrane proteins through its C-terminal region, thus potentially serving as a linker between the membrane and the cytoskeleton to recruit signaling proteins to the vicinity of sites of actin polymerization and remodeling [26].

In a steadily migrating lamellipodium, the actin meshwork remains essentially constant in breadth indicating a balance between assembly at the front and disassembly at the rear. Protrusion and retraction rates can be regulated at the level of actin assembly, apparently through the recruitment or dissociation of regulatory scaffolds. Disassembly is thought to be achieved by proteins of the ADF/cofilin family and possibly severing proteins like gelsolin, probably in cooperation with factors that break filament crosslinks [26].

Myosins might act by carrying cargo to the plus-ends of actin filaments, thus acting as cofactors in protrusion. Both myosin V and myosin VI localize to the lamellipodia of human carcinoma cells after stimulation with epidermal growth factor. Myosin V has been generally implicated in vesicle transport, but a role in protrusion has also been suggested. Myosin X localizes to lamellipodia and to the tips of filopodia in epithelial MDCK cells. It is possible that some adhesion proteins are incorporated into adhesion sites by first targeting to lamellipodia and filipodium tips, through myosin. Subsequently, complex formation and linkage to retrograde flow

could transport these components to the base of lamellipodia and filopodia, to initiate adhesion. This route is suggested by the dual localization of proteins such as VASP, talin, and integrin $\alpha 6\beta 1$ at or towards the front of the lamellipodium, as well as in adhesion complexes [26].

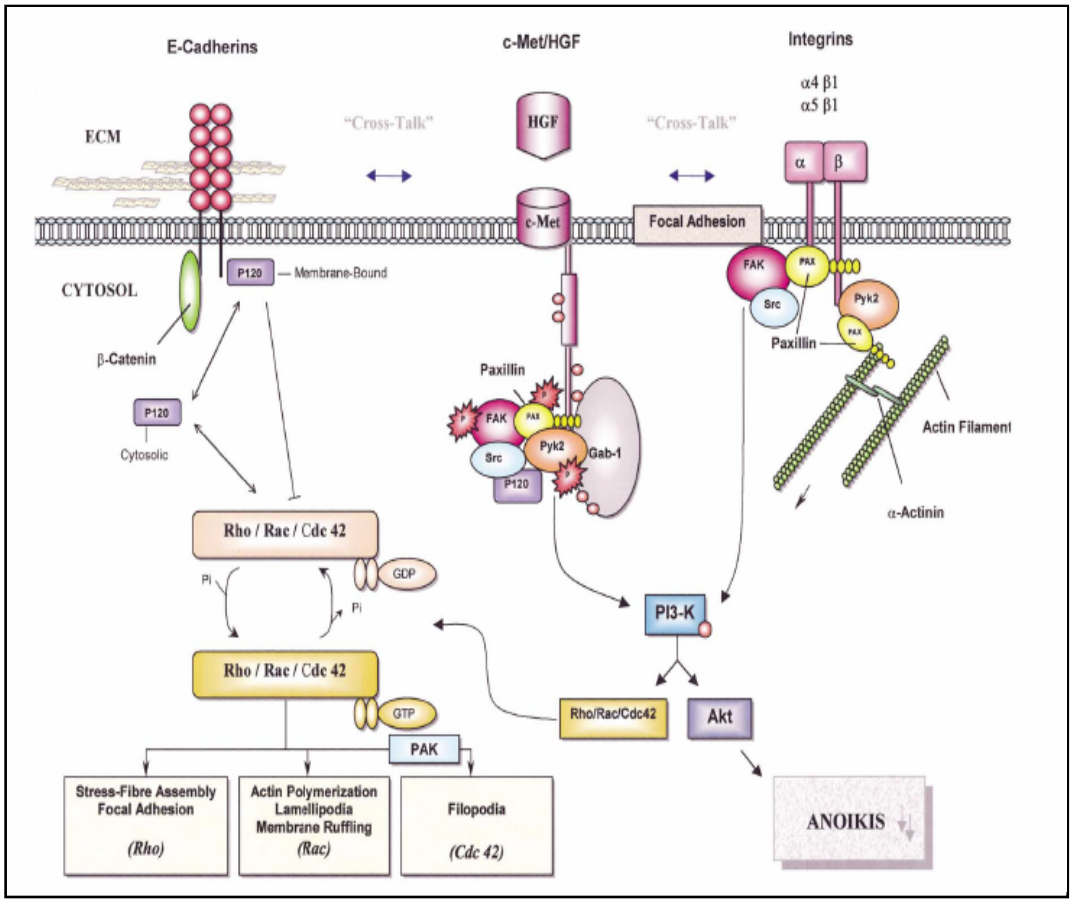
Another signaling pathway implicated in lamellipodium formation involves the p21-activated kinase (PAK) protein family. These serine/threonine kinases were identified as direct downstream effectors of Rac and Cdc42. PAKs are engaged in multiple signaling pathways, some of which might be coupled directly to lamellipodium protrusion. For instance, PAK interaction with Cdc42/Rac increases the levels of phosphorylated myosin light chain (MLC) thought to be required for the anchorage of lamellipodia. In addition, PAKs were shown more recently to activate Lim kinases to phosphorylate and thereby block the severing/depolymerizing activity of cofilin, which is proposed to effect lamellipodium turnover [26].

5.4. Adhesion sites [33,34]

Integrins are primary sensors of the extracellular matrix (ECM) environment and are thus essential for cell migration, growth, and survival. As transmembrane receptors, integrins recognize and bind to specific ECM ligands and, cooperating with signals coming from other cell surface receptors, transduce signals leading to the activation of intracellular signaling pathways and the assembly of actin-based adhesion structures that propagate cellular forces. Binding of proteins such as α-actinin and talin to integrin cytoplasmic tails, and the subsequent recruitment of the actin-binding protein vinculin and modulators of actin dynamics, are important steps in linking adhesion complexes to the actin cytoskeleton. Integrins also regulate signaling pathways to members of the Rho family of small GTPases and contribute to the dynamic turnover and remodeling of adhesion complexes by activating the focal adhesion kinase (FAK) and Src protein tyrosine kinase axis of signaling proteins, which includes the cytoskeletal regulator paxillin. Inhibition or loss of components in this pathway severely restricts adhesion complex turnover and inhibits integrin-dependent functions, such as cell migration.

Most of the proteins that build up the focal complexes share structural and scaffolding function for the integration of inward and outward signalling. Paradigmatic is the case of Paxillin, whose principal function is the integration and

dissemination of signals from integrins and growth factor receptors to effect efficient cellular migration. Motility is a complex multistep process that requires the coordination of membrane trafficking and the reorganization of the actin and tubulin cytoskeleton networks to realize net cellular movement. The activities of several p21 GTPase families which are critical to this process, are regulated trough paxillin phosphorylation and multipotent associations.



<u>The regulation of cell motility is the result of a complex signaling network deriving from cell-cell contacts, surface tyrosine kinase receptors and cell-matrix adhesion</u>.
From Maulik *et al.*, Cytokine & Growth Factor Rev. 2002 (Ref. 24).