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Invadosomes – shaping actin networks to follow mechanical cues

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1. ABSTRACT

Invadosomes are actin-based protrusions formed by cells in response to obstacles in their microenvironment, especially basement membranes and dense interstitial matrices. A versatile set of proteins controls assembly and dynamics of the actin networks at invadosomes and adhesive molecules link them with the extracellular matrix. Furthermore, polarized delivery of proteases makes invadosomes degradative. Therefore, invadosomes have been classically viewed as specialized protrusions involved in cell migration and remodeling of the microenvironment. Recent discoveries have considerably broadened this picture by showing that invadosomes respond to traction forces and can self-organize into dynamic arrays capable of following the topography of the substrate. Although these findings suggest that invadosomes may function as mechanosensors, this possibility has not been critically evaluated. In this review, we first summarize the organization and dynamics of actin in invadosomes and their superstructures with emphasis on force-production mechanisms. Next, we outline our current understanding of how mechanical cues impinge on invadosomes and modify their behavior. From this perspective, we provide an outlook of the outstanding open questions and the main challenges in the field.

2. INTRODUCTION

Invadosomes are column-like protrusions of eukaryotic cells that are built and maintained by the actin cytoskeleton (Figure 1A). In contrast to other actin-rich cell protrusive organelles like sheet-like lamellipodia, cylinder-like lobopodia and spike-like filopodia, the invadosomes are able to degrade components of extracellular matrix through localized accumulation and secretion of membrane-bound and soluble lytic enzymes, mainly metalloproteases. Different cells utilize this degradative capacity for different purposes and therefore create invadosomes with slightly different properties. Invadosomes can also be found arranged in groups of varying number and shape. These higher-order structures, such as clusters, circular arrays (rosettes) or belts, show collective behavior and properties that go beyond those of single invadosomes. A classic example of a higher-order structure of invadosomes is the sealing zone of osteoclasts. These cells organize belts of tightly packed invadosomes at their periphery in order to confine resorption of the underlying bone to a precisely defined space, called lytic lacuna (reviewed in (1)). A more recently described example are invadosome rosettes assembled by endothelial cells, which enable these cells to breach the underlying basal membrane and mediate sprouting of new blood vessels (2).

Although invadosomes are divided into two groups, invadopodia and podosomes, this distinction can

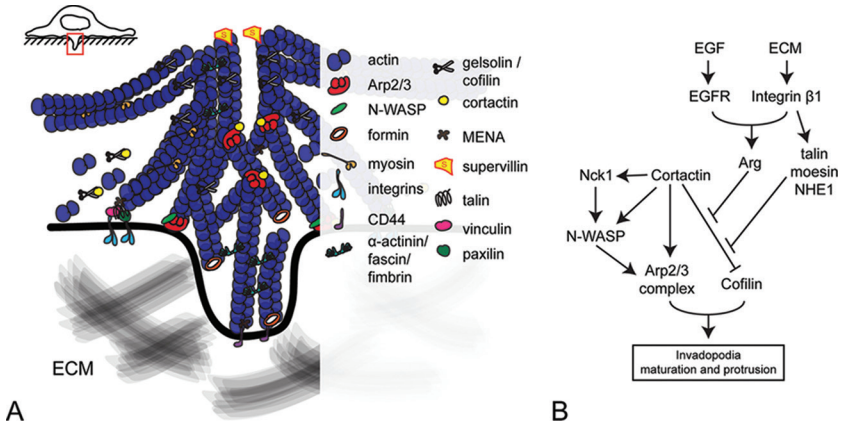


Figure 1. A. Schematic representation of selected actin-interacting proteins that form and remodel invadosomes. B. Epidermal growth factor (EGF)- and integrin-mediated signaling cascade for the production of invadopodia (35,53,54,83). Concomitant activation of EGFR and Integrin β1 activates Arg kinase, which phosphorylates cortactin. Phosphorylated cortactin facilitates the recruitment of N-WASP through NCK1. Moreover, cortactin binds to N-WASP and this complex activates the Arp2/3 complex thus leading to the polymerization of branched actin filaments. In addition, phosphorylated cortactin no longer inhibits its binding partner cofilin. Talin-mediated recruitment of moesin and NHE1 increases the pH at invadosomes which further promotes cofilin activation and ensuing formation of new barbed ends, which stimulate actin polymerization and maturation of invadosomes. See text for details.

be more readily justified by historical reasons, rather than by real functional differences. Invadosomes came to the attention of the community disguised as circular rosettes observed in Rous Sarcoma Virus (RSV) transformed fibroblasts (3). Subsequently, they were identified in other cell types and referred to as actin foci in macrophages (4) and short protrusions forming rosettes in osteoclasts (5). The name 'podosomes' appeared for the first time in the context of RSV-transformed cells (6) and subsequently acquired a rather loose meaning encompassing also the invadosome structures formed by malignant B lymphocytes of chronic lymphocytic leukemia (7). The nomenclature became more complicated since 1989, when the term 'invadopodia' was coined to describe the structures that were previously called podosomes in RSV-transformed cells (8,9).

In an attempt to reach a consensus in the field, many authors started to classify as podosomes the invadosomes formed in non-malignant cells, and as invadopodia the degradative structures of cancer cells (10,11). In this way, podosomes have been found in dendritic cells (12), endothelial cells (13) or smooth muscle cells (14), while invadopodia were found in breast (15), melanoma (16), astrocytoma (17), head and neck (18), bladder (19) cancer cells, among others. Sometimes, it is emphasized that invadopodia are smaller, more protrusive and have a longer lifespan than podosomes, and are found only in a small number (<10) in a single cell. On the other hand, podosomes are abundant, contain a prominent adhesive ring and form higher-order structures like rosettes or belts (10,20). Despite the differences outlined above, podosomes and invadopodia share many functions and most of the components. Collective terms such as 'podosome type adhesions' (PTA) (10) or invadosomes (20–22) have been proposed to emphasize this fact. Nowadays, the term invadosomes is also exclusively used for the degradative structures observed in Src-transformed cells, which are difficult to classify as either normal or cancer cells. However, it should be noted that invadopodia and podosomes not always possess all the characteristics outlined above. For example, it has been reported that podosomes can protrude when cells bearing them are put on pliable substrates (23,24). Certain cancer cell lines, such as in neuroblastoma, have been shown to produce a high number of invadopodia (25). Furthermore, the adhesive ring, once a domain of podosomes, has been visualized in the invadopodia of cancer cells attaching to a new substrate (26,27). On the other hand, some authors describing invadosomes in new cell systems chose not to follow the 'invadopodia in cancer and podosomes elsewhere' rule of thumb. For example, invadosomes in the non-malignant anchor cells of *C. elegans* go as invadopodia (28). Other authors chose not to take sides and report invadosomes on neuronal growth cones (29). While the controversy about nomenclature is far from being settled, it is perhaps more important to establish a clear distinction between individual invadosomes and their higher-order arrangements. Although these circular arrays of invadosomes are often referred to as podosomes, they have both different functions and regulatory mechanisms as compared with single podosomes (30). To avoid any confusion arising from nomenclature issues, we will use the term invadosomes to describe the general characteristic of these structures, and invadopodia, or podosomes, when referring to distinctive features.

To push, to grip, and to degrade – these three key functions of invadosomes are encoded in their different zones. The source of protrusive power of

invadosomes is the massive actin polymerization located within their inner core. Surrounding this actin-rich core is so-called invadosome ring, a circular domain enriched in adhesive proteins like vinculin, zyxin and paxillin. Interestingly, the adhesive rings may turn out to rather consist of a set of sub-domains, as recently suggested by super-resolution studies (31). The mechanical attachment to the extracellular environment is provided by integrins and other receptors for matrix components, such as CD44 (32,33). Finally, focal degradation of the extracellular matrix takes place primarily in the invadosomes due to targeted delivery of vesicles transporting matrix-degrading enzymes. Importantly, "to push, to grip, and to degrade" is a functional definition of invadosomes, and thus independent of their exact molecular make-up. For example, anchor cells of *C. elegans* display *bona fide* invadosomes despite the fact that Nematodes do not possess orthologs of major mammalian invadosome components, such as cortactin, Tks4, Tsk5 and MT1 (34). Thus, convergent evolution may have reinvented the same functional structures to fulfill fundamental cellular and organismal needs more than once.

In the recent years multiple studies have addressed the function(s) and molecular makeup of invadosomes in a variety of physiological and pathological processes. Moreover, several reviews have described the molecular composition of invadosomes (35-37), the different stages of their life cycle (38), their role *in vivo* (39,40) as well as in specific cell types (1,30,41). By contrast, the mechanisms regulating how form and function of invadosomes follow the mechanical properties of the environment and the putative role of invadosomes as mechanosensors have received much less attention. In this review, we focus on these two emerging topics in the invadosome field and summarize our current understanding of how cells generate force within invadosomes to mechanically remodel the extracellular matrix and dynamically respond to its physical properties. As the polymerization of actin into filaments produces force within invadosomes, we will first make an inventory of the actin-regulatory proteins and mechanisms that are at play within invadosomes. Then, we will describe the mechanical interaction between single and higher-order invadosomes with their microenvironment. As the term mechanosensor has been often used in the invadosome field to indiscriminately define both the elements that respond to and those that sense the mechanical properties of the ECM, we feel that it is important to bear in mind that a sensor is a device that detects or measures a physical property and records, indicates, or otherwise responds to it. Hence, we will also critically review the evidence suggesting that invadosomes truly sense the mechanical properties of the ECM. Finally, we will highlight the outstanding open questions and the main challenges in the field.

3. FORCE GENERATION – ACTIN NETWORKS OF INVADOSOMES

Each invadosome, despite its limited size, represents a self-standing actin-based protruding organelle. Molecular components that make up an invadosome include proteins regulating actin dynamics, Rho family of small GTPases, components of the adhesome, motor proteins, kinases and phosphatases, and proteinases (42). The core of the invadosomes is composed mainly of filamentous actin (F-actin) and a wide variety of actin assembly factors and actin remodeling factors, including actin bundling and severing proteins, have been shown to act in concert to ensure a high level of structural plasticity and a dynamic behavior to this region (42). Although invadopodia can persist up to several hours, actin in the invadopodia core is rapidly turned over; even in the relatively short-lived podosomes of macrophages (2 - 12 minutes), actin is exchanged two to three times before the structure disassembles (35). The rapid turnover of actin is triggered by mechanical and/or chemical external cues and involves highly choreographed activities of many actin-regulatory proteins that control the formation, maturation and function of invadosomes. Below, we outline the key proteins and pathways regulating actin dynamics within invadosomes.

3.1. Polymerization of actin at the base of invadosomes

Actin-related protein (Arp) 2/3 complex is the most extensively studied actin assembly factor of invadosomes. The Arp2/3 complex consists of two actin-related protein (Arp2 and Arp3) and five additional subunits arranged in a stable, intrinsically inactive protein assembly. Upon activation, the Arp2/3 complex binds the side of an actin filament and catalyzes the polymerization of an F-actin branch with a typical angle of about 70°. As a result, Arp2/3-complex-mediated actin polymerization generates the dendritic F-actin network found at the base of the invadopodia (43) and the Arp2/3 complex is required for the formation of invadosomes (44,45). The mechanism of activation of the Arp2/3 complex involves two co-factors, namely a pre-existing actin filament and a nucleation promoting factor (NPF) (46). Although all NPFs bind and activate the Arp2/3 complex, they can be grouped in two different classes according to the presence of some distinctive domains (reviewed in (46)). Class I neural Wiskott-Aldrich syndrome protein (N-WASP) and class II cortical actin binding protein (cortactin) are found in the core of invadosomes where they may synergistically activate the Arp2/3 complex (47-50). Moreover, both classes of NPFs are required for invadosomes formation and subsequent matrix degradation (44,51-54). Besides promoting nucleation, N-WASP and cortactin also act as adaptors for the recruitment of proteins regulating actin dynamics at invadosomes, such as cofilin and gelsolin.

Like the Arp2/3 complex, N-WASP rests in an inactive conformation attained through an intramolecular interaction between its Cdc42/Rac interactive binding (CRIB) region and the VCA domain, which is located in the C-terminal region of all class I NPFs. The VCA domain consists of a verprolin homology domain (V) (also referred to as WASP homology 2 (WH2) domain (W)), a connector region (formerly known as cofilin homology domain) (C), and an acidic region (A) and functions as minimal tripartite element to activate the Arp2/3 complex (55). N-WASP auto-inhibition is released upon binding of activated Cdc42 to the CRIB region that releases the VCA domain, which in turn binds to and activates the Arp2/3 complex (56). Not surprisingly, Cdc42 was also shown to be required for invadopodium formation and increased N-WASP activity was observed at the base of invadopodia (57). Interestingly, downregulation of Cdc42 inhibits invadopodium formation more dramatically than that of N-WASP and the Arp2/3 complex, suggesting the existence of Cdc42-dependent pathway(s) controlling the formation of invadopodia that do not involve either N-WASP or the Arp2/3 complex (44). Nonetheless, biochemical studies have shown that N-WASP could also accelerate actin polymerization from highly clustered filament barbed ends in an Arp2/3-complex-independent manner (58). Thus, N-WASP might also contribute to invadosome development in an Arp2/3 complex-independent manner by directly associating with tightly packed barbed-end clusters at the invadosome tip to promote filament elongation and ensuing invadosome extension. Moreover, given that N-WASP is recruited as one of the first proteins at sites where invadosomes form (57,59), it might function to prime those sites to initiate invadosome assembly. The fact that depletion of N-WASP reduced invadopodium formation more potently than the loss of Arp2/3 complex (17) lends support to the postulated Arp2/3-complex-independent function(s) of N-WASP.

Cortactin appears early in invadopodia, even before the onset of actin polymerization (60-62) and its phosphorylation by Src and Arg kinases is a key regulatory step controlling both the formation and the maturation of invadopodia. In fact, phosphorylation enhances cortactin's ability to stimulate N-WASP-Arp2/3 complex-mediated actin polymerization (62-64). In addition, phosphorylation of cortactin promotes the recruitment of N-WASP, Nck1, WASP-interacting protein (WIP) and cofilin to invadopodia and is required for the generation of functional invadopodia, matrix degradation and invasion in tissue culture (51,61,65-67), and metastasis *in vivo* (68).

WIP interacts with Nck1, N-WASP and cortactin and stimulates Arp2/3 complex-mediated actin polymerization (69-71). Of note, WIP promotes Arp2/3 complex-dependent actin polymerization by activating N-WASP in synergy with Cdc42 and phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$), whereas WIP on its own has been found to inhibit Cdc42-induced activation of N-WASP (69). Yet, it has recently been proposed that WIP also attains N-WASP-independent roles in invadopodium formation and maturation (67).

Two actin severing proteins, gelsolin and cofilin, have been shown to be involved in invadosome formation. Gelsolin is one of the most potent actin severing proteins and cuts actin filaments with almost 100% efficiency (72). Yet, gelsolin seems to have a cell-type-specific action as it is indispensable for the assembly of podosomes in osteoclasts (73), but not for that of the invadopodia in cancer cells (74). By contrast, cofilin has been ascribed a more general role.

Cofilin is an actin filament severing protein promoting actin polymerization and controlling the direction of cell motility (75). It was initially proposed in the dendritic-nucleation/array treadmilling model for the generation of lamellipodia that cofilin promotes the depolymerization from pointed ends and perhaps also the debranching of actin filaments in the proximal, older part of these protrusions to replenish the G-actin pool and sustain actin polymerization taking place in the distal part, namely the leading edge (76). However, other studies suggested that cofilin initiates branched actin polymerization and membrane protrusion as its F-actin severing activity might create free barbed ends that upon rapid elongation will be amplified by the Arp2/3 complex. This notion is supported by the observation that local activation of caged cofilin leads to the protrusion of lamellipodia (77). However, it should be noted that the photoactivation procedure affected a cellular area with a diameter of three micrometers rather than the narrow, nanometer-sized region close to the plasma membrane. In spite of this shortcoming, the observation that RNAi-mediated depletion of cofilin in rat breast cancer cells resulted in the formation of small, short-lived invadopodia with impaired matrix degradation has been interpreted as evidence that cofilin initiates Arp2/3-complex-mediated actin polymerization (44). Nevertheless, mounting evidence challenges this model and shows that it may not be universally applicable: both knockdown and chromophore-assisted light inactivation (CALI) of cofilin in mammalian cells resulted in enlarged lamellipodia and ruffles accompanied by increased F-actin levels. Along the same lines, depletion of the cofilin ortholog in the anchor cells of *C. elegans* resulted in massive accumulation of F-actin and loss of functional invadosomes (78). Taken together, these new data suggest that cofilin may function primarily as an actin-depolymerizing factor that shapes actin networks. Consistent with this view, the actin nucleator mDia1 was recently shown to promote the formation of lamellipodia and ruffles by polymerizing linear actin filaments mediating initial activation of the Arp2/3 complex (79), whereas the knockdown of cofilin in the same cell line did not have any inhibitory effect (T.I. and M.I., unpublished results). Thus,

the following alternative model would reconcile the above observations with the fact that cofilin is necessary for actin polymerization at invadosomes: as cofilin acts primarily on aged actin filaments (62) that are located far from the plasma membrane where activation of the Arp2/3 complex takes place, F-actin severing by cofilin may actually favor the recycling of actin monomers rather than the Arp2/3-complex-mediated amplification of the resulting barbed ends (62). In light of the above controversies, the generation of a cofilin-activity biosensor coupled with live-cell super-resolution microscopy would be instrumental in elucidating the precise contribution of cofilin during invadosome formation and maturation in space and time. Whatever the case, these studies collectively suggest that the role of the severing activity of cofilin is likely context-dependent and thus requires further investigation.

At invadopodia, the activity of cofilin is regulated by two main mechanisms, binding to cortactin and PIP_2, and phosphorylation. Direct binding of cofilin to cortactin has been shown to inhibit the severing activity (62). Interestingly, cortactin phosphorylation relieves this inhibitory effect without affecting the cofilin-cortactin interaction. However, it is unclear whether the phospho-cortactin-cofilin complex exhibits F-actin severing activity in cells. By contrast, sodium-hydrogen antiporter 1 (NHE1) mediates the release of the cofilin-cortactin inhibitory interaction by locally increasing the pH of invadopodia. As recruitment of NHE1 to invadopodia relies on cortactin phosphorylation and talin (80,81) and the inhibitory cofilin-PIP_2 interaction is partially relieved at elevated pH (82), it seems unlikely that the pool of cofilin bound to phospho-cortactin is active in cells. Besides being regulated by cortactin, cofilin is inactivated by phosphorylation at serine 3 by LIM-kinases (LIMK) and testicular protein kinases (TESKs) (reviewed in (83)). Interestingly, there appears to be an invadopodium-specific cofilin-regulatory mechanism that relies on the Rho GTPase RhoC and its downstream effector Rho-associated protein kinase (ROCK) (84). In fact, it has been recently shown that RhoC activation by the guanine nucleotide exchange factor p190RhoGEF activates the ROCK-LIMK pathway and results in the phosphorylation and inactivation of cofilin around the invadopodium cores. On the contrary, cofilin is active inside the invadopodium core as RhoC is deactivated by the resident p190RhoGAP. Of note, loss of RhoC increased the degradative capacity of invadopodia thereby suggesting that cofilin activity promotes ECM degradation (84). However, the same cells also showed reduced invasive abilities thereby highlighting that actin dynamics and the degradative capacity must be properly orchestrated to promote invasion.

3.2. Polymerization of actin at the protruding tip of invadosomes

The Arp2/3 complex is highly enriched in invadosomes but it does not localize to their protruding tips (44,45). Consequently, bundles of unbranched F-actin predominates over the branched actin network at the invading front of invadosomes (43). In comparison to the dendritic actin network found at the base of the invadosomes, relatively little is known about polymerization and organization of linear actin filament at invadosome's distal end. Nevertheless, Formins and tandem actin monomer binding proteins, actin nucleators that catalyze the formation of linear actin filaments, are thought to play a pivotal role in the polymerization of actin at the tip of invadosomes.

Formins function as dimers that nucleate and processively elongate linear actin filaments while remaining attached to the barbed end (85). Among the Formin-family proteins, Diaphanous-related formins (DRFs) have been linked to the formation and maturation of invadosomes. In particular, mammalian homolog of Diaphanous (mDia) 1-3 and Formin homology domain-containing proteins 1 (FHOD1) act downstream of Rho GTPases, and have been shown to be required for the formation of invadopodia in several cancer cell lines (43,86-88). While both mDia1 and mDia2 may accumulate within the invadosomes of breast cancer cells expressing constitutively active Src (88,89), FHOD1 did not localize to the invadopodia of oral squamous carcinoma cells (86). Interestingly, tumor suppressor $TIS21^{/BTG2/Pc3}$ was recently shown to abrogate invadosome formation by downregulating the mRNA levels of mDia1-mDia3 through the activation of Akt1 (90), adding another layer of complexity to pathways regulating actin dynamics at invadosomes. Importantly, some Formins also display actin bundling capacities, which may mediate the reorganization of single invadosomes into high-order structures (see section Mechanical support for invadosomes). Although a growing body of evidence supports the importance of Formins in the making of invadosomes, the mechanism(s) whereby Formins contribute to the formation and maturation of invadosomes have not been fully addressed. In particular, future studies should assess the relative contribution of the actin- and microtubule-regulatory activities of Formins and whether the Formins that do not reside within invadosomes, such as FHOD1, affect the life cycle of these actin-based protrusions by means of unconventional mechanisms. In this perspective, it is noteworthy that mDia1 has been shown to regulate both the cytosolic and the nuclear F-actin/G-actin ratio and its effects on gene transcription (91), whereas mDia2 seems to promote p53-dependent gene transcription in an actin-independent manner (92).

One of the tandem actin monomer binding protein, Spire-1, localizes at invadosomes in fibroblasts expressing constitutively active Src (89). Given that cooperation between different actin nucleators appears to emerge as a common strategy to regulate actin dynamics (79,93), it is not surprising that the overexpression of Spire-1 in those fibroblasts potentiated

the invadosomes' ECM-degradation activity, whereas its downregulation had opposite effects (89). Interestingly, Spire-1 may be recruited to invadosomes as part of a multimeric complex including Src, mDia1 and actin (89), raising the hypothesis that co-recruitment could dictate the cooperative action of specific pairs of actin nucleators. In addition, Spire-1 was shown to interact also with the small GTPase Rab3A, a key regulator of exocytosis that is present in the invadosomes. As matrix metalloproteases (MMPs) are among the cargoes transported on exocytic vesicles, Spire-1 might enhance invadosome-dependent matrix degradation by favoring the polarized delivery of MMPs (89). As Formin 2 (FMN2) has also been shown to partner with Spire in regulating actin polymerization and vesicular trafficking (94,95), it would be important to assess whether the mDia1-Spire-1 complex has an invadosome-specific role or rather a more general function. Anyway, the effects of Spire-1 on actin dynamics at invadosomes were not systematically analyzed (89) and whether or not Spire-1 has an actin-dependent role in the invadosomes requires further evaluation.

3.3. Remodeling of actin in invadosomes

Although site-restricted actin polymerization close to the plasma membrane creates an actin-rich micro-domain, only actin-bundling and crosslinking proteins can provide the newly polymerized F-actin with sufficient mechanical stability and the proper spatial organization needed to support invadosome function.

Invadosomes were originally discovered in Src activated cells and were characterized as structures containing actin and α-actinin (3). α-actinin is an actin cross-linking protein that produces a loosely packed network of F-actin and is recruited at podosomes with kinetics that closely follow those of actin (96). In general, the binding of α-actinin to F-actin and that of proteins inducing much tighter bundling of filaments are mutually exclusive. However, invadosomes seem to be built of different actin networks thereby providing a reasonable explanation for the presence of also tight actin bundlers like fimbrin (97,98), supervillin (74) and fascin (99). Whatever the case, the role of α-actinin is not unique because filamin, which also stabilizes the dendritic actin networks, has been recently implicated in the regulation of invadosomes. In fact, filamin was found in the actin-rich, invadosome-like structures located at the periphery of highly aggressive prostate cancer cells by means of a proteomic approach (100). However, the identity of these actin-based structures as genuine invadosomes needs to be confirmed using specific invadosome markers.

Given that the mechanical force produced by actin polymerization in the core needs to be directed and focused to sustain the protrusion of invadosomes, F-actin bundling is important for the extension of invadosomes into the ECM. In fact, depletion of fimbrin in cancer cells reduced the penetration length of single invadopodia without affecting the number of invadopodia per cell (43). In the same vein, fascin was shown to play a pivotal role in both the formation and the stability of invadopodia. Moreover, cells depleted of fascin showed also reduced matrix degradation as a result of a shorter invadopodium lifetime (43,99,101). Interestingly, fascin and fimbrin may be implicated in the same regulatory pathways as simultaneous depletion of both proteins had no additive effects on invadopodium dynamics as compared to the knockdown of either protein (43). Altogether, these data suggest that bundling of actin oversees some of the fundamental features of the invadosomes (*i.e.* lifetime, matrix penetration and matrix degradation) that enable these protrusive organelles to exert their pathophysiological functions. In addition to fimbrin and fascin, proteins belonging to the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of actin filament elongators and bundlers (102,103) participate in the regulation of invadopodia (104). In particular, a cancer-specific isoform of mammalian Enabled (MENA), referred to as $MENA^{INV}$, has been shown to stimulate EGF-induced invadopodium formation and cancer cell invasion more potently than the isoform expressed in normal cells (104). Importantly, MENA binds to and cooperates with mDia2 to regulate filopodial dynamics (103). However, the potential interplay between these two actin-regulatory proteins in invadosome formation and maturation has yet to be explored.

Profilin is an actin-specific nucleotide exchange factor that replenishes the pool of ATP-bound G-actin for filament growth and also prevents spontaneous self-assembly of actin monomers (105). Although it has been recently shown that depletion of profilin stimulates the maturation of invadosomes (106), it should be noted that its mechanism of action seems to be indirect. In fact, the effects of profilin may be related to its PIP_2 binding abilities (107) as depletion of profilin increases the availability of free $PI(3,4)P_2$ within invadosomes thereby favoring the recruitment of Tks5. In addition to that, knockdown of profilin may also increase Arp2/3 complex-mediated actin polymerization and concomitantly decrease the activity of Formins and Ena/VASP family proteins (108).

3.4. Signaling cascade for the formation and maintenance of invadosomes

Over the last few years, the signaling cascade of epidermal growth factor receptor (EGFR)-induced formation of invadopodia has been dissected (Figure 1B): when both the EGFR and integrin β1 are active, they induce the activation of non-receptor tyrosine kinase Abelson-related gene Arg, which phosphorylates and turns on both cortactin and N-WASP (109). These events ignite N-WASP-Arp2/3 complex-mediated actin polymerization and promote the formation of invadopodia (109). Moreover, activated Arg was also shown to recruit NHE1 through the integrin-actin crosslinking protein talin (80).

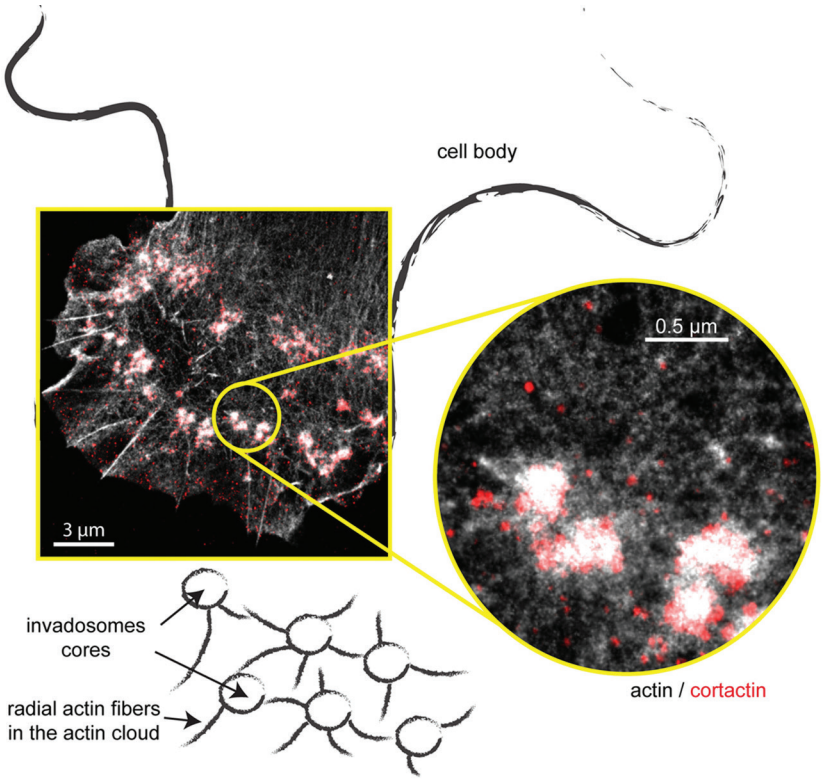


Figure 2. Invadosomes within a rosette are interconnected by a network of radial fibers. Super-resolution image shows a rosette of invadosomes (grey – actin, red - cortactin) in a mouse embryo fibroblast expressing active c-Src (SrcY530F). Zoom-in image shows a group of cortactin-rich invadosome cores connected with each other by a dense matrix of actin fibers parallel to the underlying substrate.

Given that talin is a mechanosensor, its interaction with α-actinin could endow invadosomes with the ability to sense the mechanical properties of the surrounding environment. As MENA binds integrin α5 and regulates the outside-in signaling of the α5β1 heterodimers (110), it is tempting to speculate that Ena/VASP proteins might have also actin-independent functions within invadosomes. It is also possible that such an actin-independent role may be a rather common property of the proteins regulating actin dynamics at invadosomes. In this regards, it has been recently shown that mDia2 controls gene expression also in an actin-independent fashion (92). Finally, it is reasonable to assume that the same or a similar pathway could also mediate the formation of podosomes in non-transformed cells. For example, other receptor tyrosine kinases may activate Src in cells that do not express the EGFR and Arg.

4. MECHANICAL SUPPORT FOR INVADOSOMES

Invadosomes contain a very dynamic, constantly treadmilling mass of actin filaments that needs mechanical support to counteract the resistance of the extracellular environment, as well as that of the plasma membrane, and to protrude. Surprisingly, different types of invadosomes appear to employ different mechanisms to protrude.

Podosomes and invadosomes in Src transformed cells seem to utilize the same type of contractile actin network that mechanically supports the protrusive activity of veil-like lamellipodia. The protrusion of lamellipodia depends on the contractile force generated in the behind lamellar region upon attachment of focal contacts and adhesions to the underlying substrate (111,112). In the podosomes, an actin-based region that surrounds the cores, referred to as the actin cloud (4), mimics the function of the stress fibers attached to focal adhesions and the focal contacts. The actin cloud is also composed of G-actin subunits, whose storage enables fast treadmilling of the core, and radial actin fibers (4,113). In this respect “actin cloud” may be considered a partial misnomer. Nevertheless, the radial actin fibers within the cloud link the apical region of invadosome cores to both the plasma membrane and other neighboring cores (Figure 2). Although myosin decoration suggests that the actin fibers are contractile, unbranched and organized in an antiparallel way (23,114), EM studies revealed that the majority of them shows the barbed end oriented towards the core (113). In analogy to focal adhesions and focal contacts, the radial

fibers are linked to the plasma membrane through elastic proteins like vinculin and talin (31,115). Moreover, these latter two mechanosensors connect the radial fibers to the extracellular matrix *via* integrins. In light of these analogies, it is surprising that FRAP experiments showed that the radial actin fibers display a dynamic behavior similar to the much thicker actin cores (35).

The growing end of the radial actin fibers is preferentially linked with the top of podosomes (also referred to as cap), which has a specific molecular make-up. So far, two proteins have been shown to be enriched in the cap of podosomes, the Formin FRL1 (116) and the actin-binding protein supervillin (117). FRL1 probably plays a role in the remodeling rather than in the polymerization of actin in the cap as RNAi of FRL1 led to larger podosomes without affecting their actin content. As the lack of FRL1 also strongly reduced the total number of podosomes, together these observations suggest that FRL1 may function as an actin bundler that is crucial for the existence of these protrusions (116). On the other hand, supervillin localizes to nascent podosomes and promotes the phosphorylation of myosin light chain, which leads to increased local contractility and disassembly of supervillin-decorated podosomes (117). It is clear that this picture is far from complete and further studies are necessary to address mechanism(s) of formation and regulation of radial actin fibers.

The radial actin fibers are particularly important for both the formation and the stability of higher-order podosome structures as they provide a means to connect individual podosomes cores to each other. The progressive rearrangement of invadosomes into clusters, circular arrays (rosettes) and finally peripheral belts (sealing zone) is iconized by maturing osteoclasts (reviewed in (1)). Not surprisingly, it is the actin cloud region that evolves drastically while podosomes are reorganized, whereas the cores remain largely unchanged. During the transition from clusters to mature sealing zones, the amount of actin within clouds surrounding single cores increases 20 times, fibers become more densely packed and start to preferentially link neighboring cores instead of ending on the plasma membrane (118).

Invadopodia achieve mechanical support for protrusion in a completely different manner. In fact, cancer cells often assemble invadopodia below the nucleus, the biggest and most rigid cellular organelle and also a limiting factor for the movement of cells in dense extracellular matrices (119). The specific localization of the invadopodia may be functionally linked to the requirement to translocate the nucleus in such dense networks. In this regard, the finding that invadopodia mechanically interact with the nucleus, causing indentation of the nuclear envelope in cells seeded on a stiff substrate, supports the above notion experimentally (27). Forces involved in this process are in the range of 1 nN and allow exerting pressures of around 20 kPa, which are in the range of, or even higher than, the stiffness of many tissues (see Table 1). In this way, invadopodia may be able to protrude not only in the right direction, namely into the extracellular matrix, but also at the places where it matters most, under the nucleus. Unfortunately, the mechanisms mediating the assembly of invadopodia in the proximity of the nucleus are largely unknown and future studies to fill this knowledge gap are much needed.

Finally, it is worth noting that invadosomes may also receive support from other cytoskeletal systems. In fact, it has been shown that intermediate filaments not only enter mature, highly protrusive invadosomes (43) but also are cross-linked to the actin filaments by plectin (87). Conversely, microtubules seem to be specifically excluded from the invadosome cores (27), although they are necessary for both the maturation of invadosomes in many cell types (43,121) and the stability of the sealing zone of osteoclasts (35). Therefore, while the stiff intermediate filaments (43,120) may provide mechanical support to the invadosomes, microtubules are most probably linked to the transport of specific proteins and vesicles.

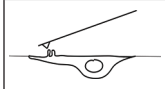
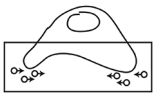
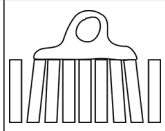
5. RESPONSE OF INVADOSOMES TO MECHANICAL CUES OF THEIR MICROENVIRONMENT

5.1. Mechanosensing

Mechanical properties of the microenvironment are as important as signals of soluble molecules for the proper execution of many cellular processes. For example, gene expression (122), cell proliferation (123), cell differentiation (124), cell migration (125,126) and degradation of the extracellular matrix by invadosomes (127) are processes sensitive to the delicate mechanical equilibrium between cells and their immediate environment. Moreover, it is not only well documented that the mechanical properties of tissues change in many pathological conditions, such as cancer (128), fibrosis (129) or atherosclerosis (130), but also that a certain mechanical property can by itself cause a disease. The observation that the density of collagen fibrils promotes mammary tumor initiation and progression perfectly exemplifies this concept (131).

For cells to respond to mechanical cues, they need to detect forces. Cells can detect external forces that simply act on the entire cytoskeleton, for example shear and stretching stresses. Moreover, they can also actively probe the mechanical properties of the microenvironment, including rigidity, by pulling on their underlying substrate and sensing by ‘active touch’ (132). To this end, cells use actomyosin-based contractility as a force-generating system to exert a probing force on the substrate. Interestingly, this system is capable to adapt

Table 1. Mechanobiology in a nutshell – definitions, numbers and selected methods.

Definitions			
Elastic modulus (Young's modulus)	Mechanical property of any given material characterizing how easily it undergoes elastic deformation under applied force		
Mechanosensor	In mechanobiology, it is a molecule or cellular structure that changes upon applied force (broad definition), or defined more exactly, it is a molecule or cellular structure that detects a physical property (force) and records, indicates or conveys its signal from the environment into a cell (narrow definition)		
Forces in a cell			
Source	**Relevant force/stress**	**Note**	**Reference**
Myosin moving on actin	3 – 10 pN	Force spectroscopy (laser trap)	(189,190)
Integrin binding fibronectin	10 – 30 pN	Optical tweezers	(191,192)
Talin unfolding	5 pN	Single molecule stretching	(193)
Vinculin connecting talin to actin	2.5. pN	FRET sensor	(194)
Focal adhesion Tension	2-5 nN/μm^2	Patterned elastomer micropost array	(195) (196)
Invadosome protruding force	1 nN (20 nN/μm^2) ~100 nN	Calculated based on nucleus identation, melanoma cells AFM, macrophages	(27) (163)
Invadosome Stress triggering degradation	~165 pN/μm^2	Single cell invasion assay (TFM measuring in z axis)	(197)
Invadosome rosette traction force	~ 0.6. nN/μm^2	6.5. kPa substrate	(166)
Smooth muscle cell pulling force	20 – 60 nN	Micropost array	(198)
Elastic modulus of selected tissues			
Tissue		**Elastic modulus (nN/μm^2)**	**Reference**
Brain		~0.3.	(199)
Fibrogranular tissue of breast		~3	(200)
Aorta		~100	(201)
Bone		~20 000 000	(202)
Most popular methods to measure forces at the cellular and the subcellular levels			
Protrusion force microscopy (PFM)		**Traction force microscopy (TFM)**	**Pliable microposts (micropillars)**
			
Atomic force microscope (AFM) follows deformation of the plasma membrane with cells on the other side		Light microscope follows fiducial markers embedded in polyacrylamide hydrogels on which cells are seeded	Light microscope follows fluorescently labeled tops of deformable micropillars on which cells are seeded
In all above methods, force is calculated based on displacement of a material of known mechanical properties. For more information see (163,203,204)			

to a wide range of conditions and it has been shown that the stiffer the matrix, the higher the intracellular tension and cell-traction forces (133). Although it was proven more than 35 year ago that a single cell may exert probing forces towards the surrounding matrix (134), there are still many open questions about the molecular mechanisms that enable cells to detect parameters like stiffness or texture of the ECM.

The best understood molecular mechanisms of cellular mechanosensing are related to cell adhesion and assign the role of probing devices to focal adhesions (FAs) (135). FAs bind firmly to the substrate through integrins and other adhesion molecules and are connected to contractile actin stress fibers traversing the cell so that any force generated within this structure is naturally balanced by the entire cytoskeleton. Although the molecular details of how force is detected are only beginning to be understood, it is well established that some proteins respond to the applied forces by stretching (reviewed in (136)). Under tension, FA proteins talin and p130Cas unfold to display otherwise hidden domains,

recruiting binding partners and thus propagating the signal. Importantly, FRET probes have allowed measuring the mechanical stress borne by these proteins and visualizing their status at the single-protein level (115). These studies revealed that signals from individual FA are integrated and processed at the level of the entire cell, rather than locally (137). In spite of the fact that the same approach could be in principle applied to the invadosomes, their putative mechanosensing properties and signal processing abilities have not yet been investigated in sufficient detail. Another well-established cellular response to applied force is opening of stretch-activated ion channels (reviewed in (138,139)). Although, several ion channels have been reported to localize to invadosomes (81,25), mechanosensitive gating of ion channels has not been observed within these structures so far.

Hence, are invadosomes genuine mechanosensors? They are generously granted this term as they share a set of tension-sensitive proteins with FAs. Moreover, the formation of invadosomes, their protrusive force and dynamic behavior are undoubtedly guided by the mechanical properties of the underlying substrate. However, a cellular mechanosensor should be a structure capable of detecting force and transducing that signal into macromolecular interactions, chemical modifications or physico-chemical secondary messengers. To our best knowledge, no direct observation of any force-sensitive protein has ever been presented to prove that invadosomes are true mechanosensors. As long as such evidence is missing, we can only discuss how invadosomes respond to the mechanical properties of the ECM.

5.2. Individual invadosomes – response to stiffness, forces and topography

It is well established that the level of tension experienced by the cytoskeleton determines the fate of adhesion structures. Nevertheless, it is also clear that different types of adhesive structures respond differently to high *vs.* low tension regimes. While FAs require tension for their formation and maturation, as mature, polarized FAs are not observed on soft matrices (135,140) or in cells with impaired actomyosin contractility (141), by contrast, the assembly of individual invadosomes is promoted by globally or locally diminished actomyosin tension.

Four lines of evidence correlate a state of low cytoskeletal tension with the formation of invadosomes. First, cytoskeletal relaxation can be promoted by a specific substrate. For example, dendritic cells (DCs) make podosomes on compliant adhesive substrates that do not support the formation of FAs (142). Moreover, fibroblasts that normally produce prominent FAs on Arg-Gly-Asp (RGD)-coated glass form instead invadosomes on fluid (RGD)-coated lipid surfaces (143). Second and at the global cellular scale, fibroblasts producing invadosome rosettes tend to lose their prominent stress fibers (3). Along the same lines, overexpression of the ion channel TRPM7 has been shown to trigger the assembly of invadosomes in neuroblastoma cells by increasing the phosphorylation of myosin heavy chain, which results in a diminished intracellular tension (25). Third and at the local scale, stimulated smooth muscle cells assemble podosomes in subcellular areas characterized by reduced contractility (144). Furthermore, many cell types produce invadosomes in the vicinity of the nucleus, where the traction forces are significantly lower than at the cell edges (145,146). Fourth and by contrast, increased tension can lead to podosome dissolution as in prostaglandin E2-stimulated DCs (147). This regulatory mechanism is at play also in macrophages where increased phosphorylation of myosin light chain, a signal that stimulates contraction, is detected in old podosomes just prior to dissolution (117).

However, the link between diminished actomyosin contractility and invadosome formation has been found in cells growing on a glass surface, a much stiffer substrate that those encountered *in vivo*. When a range of more compliant substrates was tested, it became clear that the creation of invadosomes is suboptimal on substrates that generate the lowest tension. When comparing side-by-side polyacrylamide surfaces in the lower kPa range (148,149) it was noted that invadopodia form more readily at the high end of this range. However, when a broader range of rigidities was tested, two optimal conditions for invadopodia production were discovered, 30 kPa and 2 GPa (150). Interestingly, these findings prove that cells can also discriminate between extremely stiff substrates, in the range of MPa and GPa. Surprisingly, it has also been observed that cells create invadopodia more readily on the softer side of basement membranes (BMs), namely at the stromal side. Thus, although invadosomes are often associated with stiff BMs (see also “Invadosomes in 2D – were we opportunistic or lucky?”), BMs may actually inhibit invadosome formation. Alternatively, this mechanism may allow cells to degrade preferentially the weakest spots of a BM thereby greatly facilitating invasion. A rather similar behavior has been observed in leukocytes traversing the endothelium: leukocytes first use their podosomes to palpate the endothelial surface (151) and then choose softer junctional regions to invade (152,153). Furthermore, it has been shown recently that sinusoidal endothelial cells in the liver form podosomes preferentially on stiff matrices (154). As fibrotic liver has an increased stiffness, it would be interesting to investigate whether and how remodeling of the liver tissue by these podosomes contributes to the onset and progression of liver fibrosis.

Individual invadosomes not only respond to the stiffness of the ECM but also to traction forces. Compelling evidence shows that the microenvironment has profound

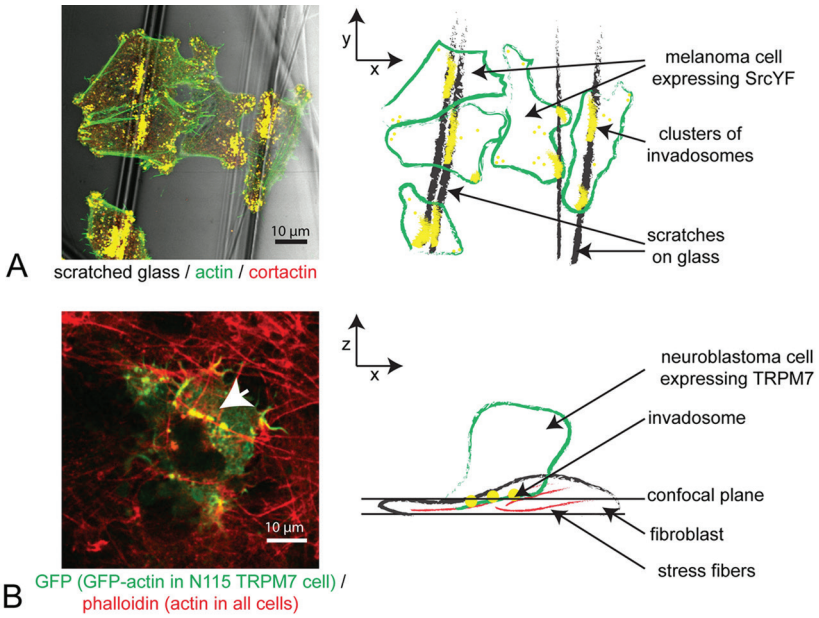


Figure 3. Individual invadosomes and clusters follow the topography of the substrate. A. A375M (melanoma) cells expressing active c-Src (SrcY530F) plated on scratched glass coverslips align their clusters of invadosomes (actin – green, cortactin – red, invadosomes visible as yellow dots and clusters) with the scratches. B. Invadosomes respond to subtle mechanical clues provided by stress fibers present in underlying cells. Neuroblastoma N1E-N115 cells expressing TRPM7 and GFP-actin were seeded atop a monolayer of Rat1fibroblasts, which form prominent stress fibers. Filamentous actin was visualized with phalloidin (red). Note that invadosomes (yellow) form in the neuroblastoma cell in correspondence to a stress fiber within the underlying fibroblast.

effects on the mode of cell migration. In particular, the mesh size of a given ECM contributes to regulating the switch between the amoeboid and the mesenchymal mode of cell migration. As the former mode of migration is poorly or not at all dependent on pericellular proteolysis, cells may readily move in an amoeboid way through the pores of the extracellular matrix above a threshold pore-size corresponding to about 10% of the cross-section area of the nucleus. Below this value, the traction forces become inhibitory and impede cell movement unless cells are capable of turning on a matrix-degradation-based motility program, such as the mesenchymal mode of cell migration (155). In light of the fundamental role of invadosomes in matrix degradation, it is not surprising that they follow the same general principle. Recent sophisticated studies showed that a cancer cell migrating into a 3D matrix pulls away from it at many points and focuses its compressive power in a single spot, which amplifies the load and maximizes the matrix-remodeling action. Only when the ECM exerts a compressive stress on this invadopodium-like structure higher than 165 Pa, the protrusion becomes degradative. Interestingly, this compressive stress at the onset of invasion seems to be independent of matrix stiffness (156). The mechanisms regulating the acquisition of the matrix-degradation abilities remain unknown, but they are likely to be similar, if not identical, to those controlling the maturation of invadosomes.

Finally, there is a body of evidence showing that invadosomes also respond to the topography of the microenvironment (Figure 3). For example, dendritic cells align their podosomes along the ridges of printed micropatterns and are able to sense ECM indentations as shallow as 100 nm (142). Interestingly, prostaglandin E2 fails to activate RhoA in DCs seeded on these stripped surfaces, which makes podosomes resistant to dissolution (142). Furthermore, different topologies of collagen seem to stimulate different types of invadosomes. Linear collagen fibers trigger the formation of linear invadopodia, special invadosome structures that rely on collagen receptor discoidin domain receptor 1 for adhesion (157) and are independent of integrin signaling (158). On the other hand, dense fibrillar collagen promotes the assembly of integrin-dependent invadopodia in breast cancer cells. Although these discrepancies cannot be explained only by the mechanical properties of the substrates, it is evident that the number of invadopodia within a single cell depends specifically on the degree of collagen crosslinking (159). As a closing remark, it is worth noting that it is often impossible to untangle the mechanical signals from the chemical ones in the context of natural matrices. For example, cells exert contraction forces on the ECM that can lead to force-regulated release of soluble signals from a latent, matrix-bound state. A prime example of that is represented by cytokine TGFß (160,161). Thus, all studies employing reconstituted and synthetic matrices to study the properties of invadosomes have as a major caveat that the observed effects might be biased by

the matrix-specific activation of a particular subset of invadosome-regulatory pathways.

5.3. Oscillating superstructures of invadosomes – response in a new scale

Higher-order structures of invadosomes are also capable of responding to the mechanical properties of the environment. Single invadosomes are linked to each other in these superstructures (see section 4, Mechanical support for invadosomes). Importantly, the structural continuity between individual invadosomes allows these superstructures to behave as single functional units endowed with emergent properties. It has been observed that the individual podosomes of a cluster undergo periodic oscillations in actin content, stiffness and pushing force, and such oscillations depend both on actin polymerization and myosin-based contractility (114,162,163). Moreover, single podosomes synchronize their oscillatory behavior with their immediate neighbors, even if they are out of synchrony with podosomes located farther than 2.5. μm (164). These findings argue against the existence of an overarching mechanism governing the behavior of the whole structure. Rather, it is likely that single podosomes within a cluster are constantly palpating the underlying surface in cycles of actin polymerization and depolymerization. The resulting pulling action exerted on the adjacent actin radial fibers, which interconnect the podosomes within a cluster, propagates the flow of information through the entire assembly and allows the emergence of a collective, locally synchronized behavior.

Individual invadosomes show a highly dynamic behavior and can rapidly disassemble and reform in a new spot. However, they do not move significantly in lateral direction and, consequently, cannot be used as a probe to drag over a surface and monitor its local properties. By contrast, circular arrays of invadosomes, referred to as rosettes, do move laterally and can potentially both sense and respond to the environmental cues in a totally new scale. Although the properties of the invadosomes that compose a rosette remain rather constant on different substrates, the rosettes move over a stiff surface faster and consist of more closely arranged cores than on a flexible substrate (165). Moving rosettes exert traction forces on a substrate comparable in value to FAs, but, in contrast to FAs, their rotation can generate also torsional traction (166). The reverse effect is also true, as exerting external force on a cell causes the displacement of invadosomes (166). Interestingly, the regulation of rosettes by tension seems to be more complicated than that of individual invadosomes. The creation of rosettes correlates with the activation of RhoA (167), a master regulator of actomyosin contractility that also localizes to rosettes (168). However, reduced actomyosin tension has been reported to have either negative (165,169) or positive (170,171) effects on the stability of existing rosettes. This sharply contrasts with the behavior of individual invadosomes, which are always stabilized upon cytoskeleton relaxation.

Due to their bigger size, invadosome superstructures have access to topographical information of the substrate at a length scale that is not available to single invadosomes. Using osteoclasts as a model system, it has been shown that when roughness of the substrate increases, rosettes become less motile, have increased stability and start to mirror the topography of the underlying surface (172). Although these rosettes, as well as the sealing zone into which they mature, cannot form on non-adhesive substrate patches, thin non-adhesive strips can be sometimes bridged by a region enriched in radial actin fibers (173). In summary, it is evident that although some general principles in the behavior of invadosome superstructures can be identified, the specific features of different cell types govern higher-order structures of invadosomes in a way that is not fully understood. A new toolbox of molecular sensors and mathematical modeling are needed to characterize in full these superstructures and their emergent properties.

5.4. Invadosomes in 2D – were we opportunistic or lucky?

Since their discovery in the early 80s, invadosomes have been mainly studied in cells plated on glass coverslips. While this setup facilitates microscopy-based experiments, it has also been the source of harsh criticisms. The long-standing question in the field was whether or not these structures would have been produced also by cells in a three dimensional (3D) environment or *in vivo*.

Due to technical challenges raised by the imaging of tiny structures *in vivo*, the ultimate proof of the existence of the invadosomes came relatively late (52,174-176). However, as our understanding of the modes of cell movement has increased with the technical advances of intravital microscopy methods (177-179), the question as to the relevance of the invadosomes in 3D had to be reconsidered. Invadosomes were, indeed, described as structures facilitating migration in 3D gels simulating the extracellular matrix (180,181). Under those experimental conditions, they appeared to be necessary for the removal of the fibers restricting cell movement (182-184). Nevertheless, invadosomes are now believed to play a major role also in 2D environments as surfaces are abundant within the body. For example, the peritoneum covering internal organs, the pleura covering lungs and thorax, and the surface of large blood and lymph vessels are recognized by cells as 2D environments and exploited extensively by cells to move (177,179). Moreover, cells need to breach tissue boundaries in a variety of physiological and pathological processes, such as intravasation, extravasation, sprouting of new blood vessels and

escape of carcinoma cells from the primary tumor. Notably, invadosomes help cells breach a 2D surface, often a dense basement membrane (2,174,176).

In this context, studying invadosomes in cells that are spread on a glass surface covered by a thin layer of extracellular matrix components does not seem to be such a bad idea. However, it should be borne in mind that the glass is much stiffer than any surface encountered by cells within the body, including bone. This and the influence that the mechanical properties of microenvironment have on invadosomes suggest that results should be interpreted with extreme caution.

6. CONCLUSIONS AND OUTLOOK

The "musculoskeletal system" of invadosomes is built and maintained by the orchestrated effort of proteins that assemble and remodel actin networks. However, it is the tension generated within the actin core that allows both single invadosomes and clusters to respond (directly or indirectly) to the mechanical properties of the extracellular matrix. Unraveling the mechanisms underlying the mechanoresponsive, and possibly mechanosensitive properties of invadosomes goes far beyond the boundaries of fundamental biology, as invadosomes are a promising therapeutic targets for new intervention strategies in diseases caused by aberrantly migrating cells, first and foremost cancer metastasis (176,185).

As presented in this review, recent findings have shed new light on how force is generated within invadosomes, as well as on how they adapt to the changing mechanical properties of the environment. We now know that the mechanical properties of a substrate define how many invadosomes will be produced in a cell, what force a single protrusive core will generate and how fast a rosette of invadosomes will move. Moreover, recent studies have revealed that invadosomes remain under tensional stress, contain tension-sensitive proteins and are mechanically coupled to the actomyosin cytoskeleton and nucleus. This work has resulted in a major conceptual advance, namely that the mechanical and topological properties of the ECM instruct the cell to deploy complex adaptive responses. The definition of invadosomes as mechanoresponsive structures is an important corollary of this breakthrough. Of note, the abilities to respond to and to sense a mechanical stimulus do not necessarily go hand in hand.

Unfortunately, direct evidence that information about the mechanical properties of the environment is received, collected and decoded within invadosomes is still missing. Thus, the fundamental question as to whether invadosomes are mechanosensors still awaits a firm answer. With regard to this point, there are three crucial questions that need to be addressed. Do talin and other mechanosensors undergo unfolding and activation in the ring surrounding the invadosome core? Which invadosome components are modified and how in order to overcome the resistance of the environment? And, what proteins are recruited in response to force, and when?

In addition, it is noteworthy that, at least in some cell types, invadosomes may be considered as self-contained (internally balanced) structures owing to the physical bonds between the core and the radial actin fibers attached to stretchable proteins. Conversely, the signals arising in the FAs become automatically available for the entire cytoskeleton, as the force in the FAs is equilibrated by the stress fibers. Does local force balance allow invadosomes to sense and process mechanical signals in a way not available for FAs? If we could generate a mechanical signal within an invadosome, would this signal act locally to control the cytoskeleton or would it be integrated at the whole cell level alike the signals originating in FAs? Along the same lines, would a stiff matrix region on one side of the cell define the behavior of the invadosomes in another region of the same cell experiencing a different stiffness? Undoubtedly, recent development of the Protrusive Force Microscopy (PFM; see Table 1) offers new opportunities to address these questions.

Beyond mechanobiology, the invadosome field still faces many challenging open questions. First, the regulation of the life cycle of invadosomes remains puzzling. How does a cell recognize that invadosomes have successfully performed their tasks and are no longer needed in a particular spot? Is the local relief of tension enough to trigger the disassembly of invadosomes? If so, what is the molecular basis whereby this signal is transduced? Alternatively, do the cleaved ECM components that are recognized by the cell as instructive signals trigger invadosome disassembly? How would such a signal be transduced and does it combine with the mechanical inputs?

Second, it is unclear whether all invadosomes are functionally equal. The complex networks formed by the actin-regulatory proteins controlling the formation of the invadosomes, along with some degree of redundancy among them, raise two questions: Do different types of invadosomes endowed with unique functions exist? If so, are they segregated temporally and/or spatially or do they rather form in close proximity? We anticipate that these issues will be progressively solved as our mechanistic understanding of the pathways regulating invadosome formation is steadily increasing.

Third and related to the previous point, the molecular mechanisms enabling cells to secrete ECM-degrading enzymes within invadosomes in response to the resistance of microenvironment need to be

elucidated (156). Again, an exhaustive mechanistic description of the pathway regulating invadosome biogenesis will be instrumental in answering this question.

Fourth, we cannot conceptualize yet how the branched actin array assembled by the Arp2/3 complex can be constrained into columnar structures like the invadosomes. The fact that Arp2/3-complex-dependent actin nucleation is usually restricted on a membrane surface clashes with the actin-rich core being encased by the actin ring, which in turn contacts the plasma membrane. Is there an invadosome-specific mechanism to activate the Arp2/3 complex? Is this shape due to spatially restricted actin polymerization or rather to the remodeling activity of actin bundlers and cross-linkers? While the spatial control of cofilin activity may play a role in this process (62,80,84), the controversies about its function (44,186) strongly suggest that much still needs to be discovered. Whatever the case, the fact that linear actin filaments are the predominant F-actin species found in the distal part of invadosomes further threats this simple model. Future research aiming at elucidating these mechanisms cannot exclude a deeper molecular understanding of the key actin regulatory proteins and interplay thereof within invadosomes and live-cell super resolution microscopy to zoom in to the topology of the actin cytoskeleton.

Fifth, untangling the mechanical and chemical signals that govern the behavior of invadosomes perhaps represents the biggest challenge in the field. As we emphasized in the section “Individual invadosomes – response to forces, stiffness and topography”, this point is an especially important issue for cells confronted with substrates like collagen, which can both polymerize in different forms defining the topography of the substrate and trigger outside-in signaling of adhesion receptors. Artificial fibrillary substrates of defined composition, architecture and mechanical properties are readily available in the field of tissue engineering (reviewed in (187,188)) and should be exploited to facilitate such studies.

Last but not least, which properties ascribed to invadosomes in tissue culture hold *in vivo*? The rapid developments in the field of super resolution optical microscopy and intravital imaging make us hopeful that studying invadosomes in living organisms at near-molecular resolution may soon provide compelling answers to this question.

In summary, the recent years have witnessed a rapid progress in our understanding of the regulatory mechanisms and biological function of invadosomes. Although there is still a long way to go before we can conceptualize all the unexpected properties and somewhat mysterious behavior of these actin-based structures, we are sure that the integration of cell biology, biophysics, advanced imaging techniques and mathematical modeling will greatly help us in this journey.

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Abbreviations: AFM: Atomic Force Microscopy; BM: basal membrane; DC: dendritic cell; ECM: extracellular matrix; FA: focal adhesions; FRAP: Fluorescence Recovery After Photobleaching; MT1: metalloprotease 1(MMP14); RGD: Arg-Gly-Asp; RSV: Rous Sarcoma Virus.

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