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Proteolytic and Mechanical Remodeling of the Extracellular Matrix by Invadopodia in Cancer

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Abstract

Cancer invasion and metastasis require remodeling of the adjacent extracellular matrix (ECM). In this mini review, we will cover the mechanisms of proteolytic degradation and the mechanical remodeling of the ECM by cancer cells, with a focus on invadopodia. Invadopodia are membrane protrusions unique to cancer cells, characterized by an actin core and by the focal degradation of ECM *via* MMPs. While ECM can also be remodeled, at lower levels, by focal adhesions, or internal collagen digestion, invadopodia are now recognized as the major mechanism for MMP-dependent pericellular ECM degradation by cancer cells. Recent evidence suggests that the completion of epithelial-mesenchymal transition (EMT) may be dispensable for invadopodia and metastasis, and that invadopodia are required not only for mesenchymal, single cell invasion, but also for collective invasion. During collective invasion, invadopodia was then shown to be located in leader cells, allowing follower cells to move via cooperation. Collectively, this suggests that invadopodia function may be a requirement not only for later steps of metastasis, but also for early invasion of epithelial cells into the stromal tissue. Over the last decade, invadopodia studies have transitioned into in 3D and *in vivo* settings, leading to the confirmation of their essential role in metastasis in preclinical animal models. In summary, invadopodia may hold a great potential for individual risk assessment as a prognostic marker for metastasis, as well as a therapeutic target.

INTRODUCTION

Metastasis, the cascade of events that leads to the growth of secondary tumors in distant organs and consequent failure of vital organs, remains the main cause for cancer-related deaths [1], [2]. The earliest step of metastasis is the invasion of cancer cells into the surrounding stromal tissue. In epithelial cancers, *i.e.* carcinomas, this invasion requires cancer cells to breach the *basement membrane* (BM), a thin and dense sheet-like layer of extracellular matrix (ECM) primarily made of laminins and collagen IV. Beyond the BM, cells navigate through a dense network of *stromal ECM*, composed primarily of fibrillar collagen I, but also containing >300 other proteins, including other collagen types, fibronectin and proteoglycans, as well as growth factors and chemokines bound to ECM components. The BM and stromal ECM compartments constitute main physical barriers to

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cancer cell invasion and migration, and are continuously remodeled as the cancer progresses [2], [3]. Remodeling of the ECM during cancer progression includes a variety of biophysical and biochemical changes in its composition (*e.g.* deposition, modification, degradation) and architecture (*e.g.* fiber aligning, bundling and reorienting, stiffening, cross-linking, pore enlargement). Changes in the ECM can be induced by cancer cells but also by fibroblasts and immune cells that are recruited to the tumor [4]–[9]. In this mini review, we will cover the mechanisms of proteolytic degradation and the mechanical remodeling of the ECM by cancer cells. For comprehensive reviews on ECM remodeling by different cell types, see [7], [10], [11].

One strategy unique to ECM remodeling by cancer cell is the assembly of *invadopodia*, cancer-cell specific structures capable of adhering to the ECM, physically protruding into it and proteolytically degrading it. The term invadopodia first appeared in 1989 when Chen *et al.* reported the punctate degradations of fluorescent fibronectin under *vsrc* transformed chicken fibroblasts [12]. Previously, such structures were reported in 1985 by Chen *et al.* [13], as well as Tarone *et al.* but referred to as *podosomes* [14]. In the current literature, "podosome" refers to closely related counterparts in non-transformed cells, whose function is also ECM adhesion and remodeling [15]. Examples of cells forming podosomes are vascular cells (endothelial or smooth muscle cells) [16] and cells of monocytic lineage (macrophages, dendritic cells and osteoclasts) [17]. Since the discovery of invadopodia and podosomes, sometimes jointly referred to as *invadosomes*, mounting knowledge has been gathered on their molecular composition, dynamics and the signaling pathways involved in their assembly and function (reviewed in [18]–[20]).

Briefly, invadopodia are membrane protrusions characterized by an actin core, sites of cell-ECM adhesion [21] and by the focal degradation of ECM *via* matrix metalloproteases (MMPs) [22]. In cells plated onto 2D matrices, invadopodia assemble on the ventral surface and can be detected by punctate degradation spots at the sites of ECM contact. The initiation of invadopodia assembly can be stimulated by soluble factors (*e.g.* growth factors, chemokines, ECM fragments, Table 1) [18], [23], hypoxia [24] or via direct cell-cell contact with macrophages [25]. The first step of assembly results in *invadopodia precursors* composed of cortactin, cofilin and N-WASP proteins associated to actin filaments (Figure 1). Precursors are then anchored to the plasma membrane *via* the scaffolding protein Tks5, followed by the recruitment of β1 integrins that bind to the ECM (Figure 1). The formation of these cell-ECM adhesion sites triggers elongation *via* actin polymerization and invadopodia maturation, during which MT1-MMP vesicles are delivered to their tip and initiate pericellular degradation of the ECM (Figure 1). After degrading the surrounding ECM, invadopodia disassemble [26], which enables cells to translocate their body and migrate to the next location where new invadopodia will assemble [27] (Figure 1), resulting in switching between invadopodia and migratory state.

Invadopodia are composed of over 50 proteins, most of which also act as components of other adhesive and protrusive structures, such as focal adhesions, lamellipodia and filopodia. However, Tks5 is one unique component of invadopodia, and hence, a unequivocal marker [28]. Importantly, each step of invadopodia assembly is reversible and dependent on the local availability of the necessary components. Therefore, not all invadopodia

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precursors will proceed to anchoring, elongation, maturation and ECM degradation. To detect invadopodia that have matured and degraded the ECM, multiple markers are required: two colocalized structural proteins (*e.g.* Tks5 and cortactin), in addition to a marker of ECM degradation.

While most early studies on invadopodia were performed *in vitro*, we now have evidence for invadopodia existence in both preclinical animal models [25], [29]–[31] and in human samples [32]. Importantly, invadopodia were shown as necessary for the metastasis of cancer cells in mice [29], [30]. In the last decade, several groups have studied invadopodia in 3D and *in vivo*, recognizing their potential as an anti-cancer therapeutic target [33]–[35].

Identification of invadopodia in 3D and in tumors

Invadopodia have mostly been studied in 2D, using cancer cells plated on top of a thin layer of isotropic, fluorescent matrix. Here, punctate localization of structural markers colocalized with negative signal of degraded fluorescent matrix provides a clean way to quantify invadopodia. However, in 3D, standardizing invadopodia imaging and detection can be challenging due to a) variability and heterogeneity of cellular morphology, b) cell directionality, orientation, and location within the matrix, c) density and architecture of the ECM. In addition, probes used for detecting ECM degradation in 3D can vary among studies, as each one of them has their own benefits and disadvantages. For example, dequenched matrix components, such as DQ-collagen [36], demonstrate fluorescent signal upon both remodeling and ECM degradation, appearing colocalized with invadopodia markers but also at sites of matrix bundling and contraction. Next, antibody against degraded collagen (C1 ¾) can appear colocalized with invadopodia and intracellularly [29]. Finally, MMP activity probes or biosensors do not provide direct evidence of completed function by MMPs [29], [37], [38].

Several studies have defined invadopodia in 3D environments or *in vivo* utilizing these different markers, in different cancer cell lines and ECM types. For example, when breast carcinoma MDA-MB-231 cells were plated on top of a thick Matrigel layer, invadopodia/ filopodia-like protrusions were observed. These protrusions were positive for F-actin, cortactin and active Src, and some of them colocalized with MT1-MMP pH fluorin, which is visible only when MT1-MMP vesicles are emptied out of the cell [39]. Tolde *et al.* found that Src-transformed rat sarcoma RsK4 cells embedded inside a pig dermis-matrix form thick protrusions positive for cortactin, phospho-cortactin and degraded collagen, which extend into filopodia-like structures devoid of invadopodial markers [40]. When plated on, or in fibrillar collagen, MDA-MB-231 form linear invadosomes that are Tks5-, F-actin- and cortactin-rich and degradative. These invadosomes are independent from integrin β1 and β3 but require the discoidin domain receptor 1 instead, which sets them apart from classical invadopodia [41]. Breast cancer cells MDA-MB-231 embedded into Matrigel, a mixture of Matrigel and collagen I, or in Geltrex, were shown to form long invasive pseudopods that contained cortactin-, Arp2/3-, N-WASP-rich puncta on their side and at their tip [21], [42], [43]. Some of the puncta were also rich in vinculin and focal adhesion kinase (FAK) suggesting that in 3D, invadopodia and focal adhesions may merge into a hybrid structure.

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Invasive protrusions were dynamic with cycles of extension and retraction through the matrix, characteristic for invadopodia [27] (Figure 1).

In Perrin *et al.*, using 4T1 breast cancer spheroids embedded in rat-tail collagen I, we observed long protrusions positive for Tks5, F-actin and degraded collagen present at the front and the sides of leader cells [44]. Other studies suggested that most degradation in 3D rat-tail collagen I is not localized at the leader tip, but in front of the nucleus, where invadopodia form a degrading belt which releases the cell from nuclear confinement [45], [46]. Studies agree, however, that the direction of invadopodia extension in 3D determines the direction of the cell translocation in the following step.

In vivo, using mouse xenografts of MDA-MB-231 [47] and MTLn3 [30] cells, invadopodia were shown to be essential for intravasation and lung metastasis of breast carcinoma. As shown by machine learning classification of microenvironment features, invadopodia are assembled in *perivascular niches*: regions adjacent to mature blood vessels, rich in macropinocytic macrophages and aligned, cross-linked collagen fibers [29]. In these niches, invadopodia appeared as 0.5-2 μm-wide, >3 μm long protrusions enriched in Tks5, actin, cortactin and N-WASP [30], and colocalized with degraded collagen. Interestingly, invadopodia were found to be assembled mainly by the cells that were in the G1 phase of the cell cycle, which were present at high density in perivascular niches [48]. Invadopodia were pointing towards the blood vessels and the surrounding collagen fibers, and cells assembling invadopodia showed relatively slow cell velocities in the same direction (0.15 μm/min). In comparison, cells exhibiting contact-guidance, which freely migrated along collagen I fibers were found to move ten times faster, at speeds of 1 μm/min. Importantly, both directly targeting invadopodia via Tks5 knockdown, or indirectly inhibiting their assembly via inhibition of collagen cross-linking led to inhibition of ECM degradation, intravasation and the formation of metastases, without affecting contact guidance. In a different model of metastasis, the chorioallantoic membrane assay, invadopodial protrusions were seen to extend through the endothelial layer, enabling the extravasation of cancer cells [31]. In this case, Tks5 knockdown also inhibited formation of metastases.

In summary, in 3D matrices and *in vivo*, invadopodia morphology and the location of pericellular degradation may vary with ECM components and cell lines. The next section will cover in more details the ECM characteristics that are associated with invadopodia assembly and the ones that do not require invadopodia for cell movement.

ECM degradation and mechanical deformation by cancer cells

In the primary tumor context, invadopodia were shown to assemble at the carcinoma stage inside the perivascular niche, facilitating cell entry into blood vessels. Perivascular regions are rich in cross-linked fibers of collagen I and in endothelial BM components like collagen IV and laminin. To mimic the pathophysiological context, most studies of invadopodia are done using cells plated on gelatin (denatured collagen I), cells embedded in reconstituted collagen I or in a mixture of collagen I and BM (Matrigel, Geltrex). While missing the original collagen I architecture, gelatin provides a homogeneous layer of ECM and enables high-resolution imaging. Gelatin can also be cross-linked, which increases its stiffness and

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results in a biphasic increase in the number of invadopodia and in the level of ECM degradation [27].

Systematic motility studies of individual cancer cells embedded in 3D collagen I have demonstrated that in relatively compliant matrix, such as pepsinized bovine collagen at concentrations <15 mg/ml, the cell contractility and the traction forces generated through focal adhesions result in mechanical deformations of the ECM, which is sufficient for the cell to move (Figure 2). In compliant **3D** gelatin, cells were also shown to move amoeboidally, *via* actomyosin contractility, which initiated shedding of β1 integrin-positive microvesicles [50]. In Matrigel, by varying matrix thickness, it was shown that compressive traction stresses >165 Pa induce invadopodia-dependent motility, while lower traction stress led to bleb-based motility, where forces are generated *via* hydrostatic pressure [51].

Increasing level of confinement compresses the cell nucleus, causing nuclear deformation and frequent ruptures and repairs of nuclear envelope (NE), which in turn may lead to DNA damage [52]. The expression levels of main determinants of nuclear stiffness, nuclear envelope proteins lamins A/C, determine nucleus deformability and consequently, the frequency of NE rupture events [53]. While a decrease in lamin A/C levels may give protease-independent cells a certain advantage, it can also cause the mechanical instability of the nucleus, potentially leading to cell death.

When pore sizes in collagen I are smaller than 7 μm^2, corresponding to approximately 1 mg/ml of telopeptide-intact, acid extracted rat-tail collagen I, or 15 mg/ml for pepsinized bovine collagen I, or 10% of the nucleus cross-section, MMP-independent cell migration is arrested [49]. Cells confined in such dense collagen networks, with pores <7 μm^2 and constricting collagen fibrils, trigger the requirement for MMP-dependent motility, with invadopodia as the primary agents [45] (Figure 2). Similarly to 2D [27], cross-linking of collagen in 3D or *in vivo*, increases the number of invadopodia and the levels of ECM degradation [29]. Recent study revealed an active role of nucleus in triggering invadopodia assembly and ECM degradation. The nucleus was shown to be directly linked to the centrosome, a connection regulated by nesprin 2. Nuclear deformation during confined cell movement acts as a mechanosensor, triggering polarization of MT1-MMP-endosomes and their release ahead of the nucleus. When the matrix pores are enlarged by MT1-MMP, forces generated by molecular motors dynein and kinesin attached to the nuclear surface act to pull the nucleus along the microtubules.

Protease-dependent motility (*i.e.* invasion) consists of Invadopodia state, during which cells remain stationary, and a Migration state, during which cells translocate [27] (Figure 1). Cells switch between these states on the scale of hours, with the switching frequency being dependent on the cross-linking levels. While in the invadopodia state, cells engage in ECM degradation and in actin polymerization-depolymerization oscillations, which happen on the timescale of minutes (7-12 minutes) [29], [43]. Such oscillations extend invadopodia length and widen the matrix deformations, but also generate forces that can be visualized by interference stress microscopy [54]. Using interference stress imaging, additional fluctuations in the forces exhibited by invadopodia were detected. These forces, appearing on the timescale of seconds, likely originate from actin treadmilling in invadopodia.

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Mathematical modeling suggests that the control of invadopodia oscillations is due to the balance of five forces: protrusion generated by actin polymerization, pull generated by retrograde flow, cell adhesion, ECM resistance and myosin contractility [55], [56].

Importantly, while the invadopodia assembly requires both chemical (*e.g.* growth factor-related) and mechanical (*e.g.* integrin-related) inputs [57], some studies suggest that under some conditions, chemical and mechanical outputs of invadopodia may act independently. For example, in MDA-MB-231 cells plated on top of fibrillar rat tail collagen I, invadopodia assembly exerts forces onto collagen fibrils, bending them [58] (Figure 2). This mechanical remodeling is powered by actin polymerization at invadopodia sites and requires MT1-MMP to be delivered to the cell–matrix contacts, however, it is independent of its collagenolytic activity.

Collagen I and BM, while physiologically relevant, are structurally and mechanically heterogeneous, making them hard to standardize, or control specific mechanical characteristics. By utilizing hybrid materials, where collagen or BM are combined with biomaterials which are easier to control (PEG hydrogels, GelMA, alginate), new aspects of mechanical remodeling by invadopodia can be assessed. One example of a hybrid biomaterial used to address invadopodia assembly consists of alginate and BM [59]. Interpenetrating networks of alginate-BM with different degrees of plasticity were created, all sharing stiffness that matched tumor tissue (1.8 kPa) and pore sizes of approximately 40 nm. While MDA-MB-231 cells remained immotile in low plastic matrices (with 10% degree of plasticity for 1 h of 100 Pa creep stress), highly plastic matrices (30%) initiated assembly of protrusions that are similar to invadopodia, and followed by cell migration. These protrusions were Tks5 enriched and oscillatory, similarly to invadopodia [28]; however, they did not exhibit any proteolysis. In these highly plastic alginate-BM networks, the matrix displacement by cell-generated forces seems to be sufficient for cell translocation. While it is not clear whether similar ECM properties and cellular behaviors can be found *in vivo*, this study elegantly illustrates plasticity of migration modes in cancer cells and their ability to move through plastic matrices with small pores without degrading them. A follow-up modeling study provided a mathematical model of invadopodia oscillations, showing that an increase in the ECM plasticity, associated to more permanent deformations, increases the potential length of invadopodia [55].

Invadopodia are recognized as the major mechanism for MMP-dependent pericellular ECM degradation by cancer cells and were shown to mechanically remodel the ECM. It is, however, important to point out that both events can occur as the result of other mechanisms. For example, in addition to exerting traction forces, focal adhesions were shown to be a site of MT1-MMP mediated ECM degradation [60]. Also, MT1-MMP was suggested to function at lamellipodia, where it is recruited *via* an interaction with CD44 [60]. Similarly, amoeboid melanoma cells in collagen were shown to degrade the ECM by secreting MMP-9 and MMP-13 [59] and to internalize collagen fragments [62]. Finally, the pericellular proteolysis by invadopodia is associated with secretion of exosomes [63] that can contain MMPs and can hence serve as long-range proteolytic agents [64].

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Role of invadopodia-mediated ECM degradation in 3D collective invasion and metastasis

While most studies of invadopodia have focused on mechanisms of single cell invasion, invasion can often be collective, *i.e.* it involves the coordinated movements of multiple cancer cells, or in some cases, cancer cells and fibroblasts, or macrophages [65]. Data so far demonstrates the essential role of *leader cells*, located at the front of the collectively invading strands or clusters. These leader cells engage in MMP-dependent degradation and/or mechanical remodeling of the ECM. Mesenchymal cells can also demonstrate collective invasion, when moving in multicellular streams with no cell-cell adhesions [66]–[70] (Figure 3).

Tumors contain multiple host cell types, including fibroblasts and immune cells, as well as heterogeneous cancer cell clonal populations, and any of them can act as leaders. In the early stages of tumor progression, fibroblasts are a major contributor of ECM remodeling at the tumor edge. When fibroblasts act as leader cells, mechanical ECM remodeling they accomplish *via* contractility-based mechanisms may be sufficient to generate the low-resistance path in the BM [69], and the stromal ECM [70]. Fibroblasts can lead collective cancer cell invasion by generating lower density ECM ahead of cancer cells and pulling them ahead, which is facilitated by heterotypic N-cadherin-E-cadherin interactions between fibroblasts and cancer cells [71]. In some cases, fibroblasts can also form invadosomes, undertaking not only mechanical, but also proteolytic remodeling of the matrix [72]. Cancer cell invasion can also be supported by macrophages, where macrophages and cancer cells engage in multicellular streams maintained by paracrine EGF-CSF1 loop [70]. In addition, in the intravasation assay, upon direct contact with macrophages, cancer cells were shown to locally activate RhoA, inducing invadopodia assembly [25]. In the extravasation assay, macrophage degradation of endothelial contacts can promote cancer cell exit from the blood vessels [73]. Further, when placed in the Matrigel surrounding cancer cell spheroid, the MMP-dependent motility of macrophages generates tunnels inside the ECM and enables cancer cells to switch from MMP-dependent to MMP-independent movement inside these tracks [62].

To better represent heterogeneous clonal populations in tumors, several recent studies turned to combining invasive and non-invasive cell lines in the same assay, which resulted in reports of a *cooperative invasion*, where invasive cells act as leaders, remodeling the ECM in an MMP-dependent fashion, for the non-invasive follower population. Cooperative invasion was demonstrated for melanoma cells injected in the zebrafish embryos [74], and using spheroids of invasive cancer MDA-MB-231 and mammary epithelial MCF10A cells in collagen I [75]. Our recent work has shown that while invadopodia is necessary for leader cell position, non-invasive cells can follow, both in the absence of MMPs and cell-cell adhesions [44]. When N-WASP is knocked down in leader cells, cooperative invasion is lost [76], while knock-down of MT1-MMP in leaders blocks collective invasion and microtracks formation [46], strengthening the active role of leader cells. In contrast, the passive role of follower cells is corroborated by the data showing that the laser-etched microtracks in collagen gels are sufficient for the collective invasion of the non-invasive MCF7 cells [77]

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and the primary cells from mouse tumors [78]. Importantly, invadopodia in leaders are also necessary for *cooperative metastasis*, which requires adherens junctions between leaders and followers, mediated via E-cadherin expression (Figure 3) [44]. While it is not yet clear how the cancer cell clusters cross the blood vessel walls, a possible mechanism could be cluster unfolding into single-file chain with leader cell at the front, similarly to how clusters unfold when traversing through capillaries [79].

In summary, introducing heterogeneity into invasion assays has established the value for studying invasion and ECM remodeling in the multicellular context, and resulted in revealing the spatial and temporal requirements for ECM remodeling. The observation of active role of leader cells in invasion poses the question of whether the leaders and followers also exist in collectives of similar cells, and if so, whether the role of leader and follower can be transient.

Plasticity of invadopodia and leader cells during collective invasion

Since most carcinoma cell lines assembling invadopodia invade as single cells and Twist 1 expression was established to be necessary for invadopodia [50], completion of the EMT is commonly regarded as a requirement for invadopodia emergence and subsequent metastasis. However, recent evidence suggests that the completion of EMT may be dispensable for invadopodia and metastasis [80], [81], as inhibition of miR-200, Twist1 or Snail does not eliminate metastases in mouse models. Further, a hybrid epithelial/mesenchymal (E/M) mammary carcinoma cell line 4T1 expresses Twist 1 [82], assembles invadopodia, invades in 3D spheroid model and metastasizes, while their isogenic mesenchymal counterpart, 67NR cell line, does not [45]. This suggests that EMT completion is not required for invadopodia emergence. Moreover, what 67NR results suggest is that depending on the specific driver of EMT (*e.g.* hypoxia, ECM stiffness, EGFR, Notch pathways etc, see Table 1) and specific transcription factors regulating EMT (Zeb, Snail, Twist, FOXC2 and GSC), the EMT process may yield slightly different EMT-associated phenotypes. This line of thinking is further strengthened by two recent, contrasting observations in E/M pancreatic and mammary carcinoma cell lines. In pancreatic BxPC-3 cells, E-cadherin co-localized and interacted with invadopodia components, and the knockdown of E-cadherin impaired invadopodia assembly and function [83]. In mammary 4T1 cells, E-cadherin knockdown did not eliminate invasive ability, but switched the invasion mode in 3D spheroids from collective to single-cell [45], suggesting E-cadherin role may be cell type-, or cancer type-specific.

Importantly, despite being capable of invadopodia assembly and even if integrins and growth factor receptors are activated, not all cells will exhibit invadopodia. We have shown that within mammary xenograft tumors made of MDA-MB-231 cells, the invadopodia phenotype was only exhibited in 15% of cells present in the perivascular niche, regions where major blood vessels were surrounded by cross-linked collagen fibers and perivascular macrophages [29]. One of the additional requirements may be intrinsic, as invadopodia degrade mainly during the G1 phase of the cell cycle and are not present in S, G2 or M phase [49]. Consequently, in spheroid invasion assay, leader cells are present in G1 phase. In order for the leader cells to progresses through the rest of the cell cycle, leader-follower switching

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occurs, with the new leader in G1 phase, and the old leader retreating back to the spheroid core [49]. Moreover, leader-follower exchange in spheroids was described to occur based on intracellular level of energy, and can be postponed by addition of exogenous mitochondria to leader cells [84]. While the evidence so far argued that the leader and follower roles are assigned based on epigenetic heterogeneity of cell subpopulations [85]–[87], these observations suggest that the leader role can be transient, and that cells can switch leader and follower positions based on the cell-cycle status and on the intracellular energy levels.

More precise dissection of the leader and follower phenotypes, including molecular requirements, but also spatial and temporal information on their emergence and the role in tumor progression and metastasis have the potential to help in developing predictive diagnostics, and in developing more specific targeted treatments against metastasis.

Conclusion

In this mini review, we focus on the role of invadopodia in 3D and *in vivo* invasion and metastasis, and discuss their contribution to the proteolytic and mechanical remodeling of the ECM during cancer invasion. We described how interdisciplinary mechanobiology approaches, spanning from cell biology and microscopy to biomaterials, microfabrication and mathematical modeling contribute to a better understanding of invadopodia and invasion, and lead us towards translational potential of invadopodia as a prognostic marker and therapeutic target.

Recent technological developments in the aforementioned fields may soon be implemented into invasion field. For example, some new biomaterials allow cell retrieval following time-lapse imaging, such as temperature-sensitive collagen [88], reversible ionic cross-linking or photopolymerization [89] of hydrogels. New approaches are also being developed to complexify invasion assays and develop tumor-on-a-chip approaches. This includes co-cultures of patient-derived cancer cells, cancer associated fibroblasts or tumor associated macrophages, pre-adipocytes, and most recently, vasculature [90], [91]. Bioprinted tumor microenvironments are now available, where patient biopsies can be inserted into a stromal mix of fibroblasts, endothelial cells and mesenchymal stem cells in alginate-gelatin hydrogel [92]. While most computational models which dissect ECM remodeling currently include a single level, they will likely soon evolve into multiscale platforms for analysis of time-dependent ECM changes, with positive and negative feedbacks from direct and indirect cell-cell interactions and more open-source options [89].

So far, most 3D and *in vivo* invadopodia research has been done using established cell lines. In breast cancer, cell lines capable of invadopodia assembly mainly come from metastatic tumors, suggesting a link between invadopodia and metastasis. According to their molecular type, cell lines capable of invadopodia assembly are mainly triple-negative (including MDA-MB-231, 4T1 etc.) and Her2-overexpressing cell lines (such as Skbr3). In contrast, ER+/PR+ cell lines (including MCF7, 67NR) do not assemble invadopodia. To test the invadopodia role in different steps of tumor progression and metastasis, dynamic intravital imaging in animal models was instrumental. In established primary tumors, invadopodia were demonstrated to be necessary for basement membrane remodeling during intravasation

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and extravasation [29], [31], [93], [94]. Even early stages of tumor progression may involve the local assembly of invadopodia. For example, TGFβ stimulation can lead to invadopodia assembly in normal mammary epithelial cells MCF10a [95]. Further, a few hybrid E/M cells with invadopodia can lead clusters of non-invasive cells during invasion [45]. Finally, a study using MCFDCIS.com intraductal xenograft suggested that the transition from ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC) requires MT1-MMP overexpression, which could potentially be delivered by invadopodia [96]. Future intravital imaging studies of DCIS-IDC transition may be able to resolve this open question.

To translate the invadopodia research into the clinic, it will be necessary to establish invadopodia detection tools in clinical samples and test if invadopodia frequency correlates with the metastatic load. To date, primary cells isolated from metastatic head and neck squamous cell carcinoma [97], glioma [98] and bladder cancer patients [99] were directly used in invadopodia assays and shown to degrade ECM in 2D. Puncta positive in Tks5 were shown to be present in human tumor surgical specimens of pancreatic adenocarcinoma [100]. In addition, in premalignant lesion biopsies, areas where cortactin, Tks5 and MT1-MMP expression were found co-localized were shown predictive of malignant transformation [32]. Inhibitors of ABL tyrosine kinase, expression of which correlates with poor prognosis and distant metastasis, were shown to inhibit invadopodia and metastasis in mouse xenografts [34]. This suggests that quantification of invadopodia frequency in surgically removed primary tumors holds a great potential as a prognostic marker for individual risk assessment for metastasis or effectiveness of neoadjuvant therapies to prevent or stop metastasis. Such analyses may in the future be followed by adjuvant treatments with invadopodia inhibitors. A number of drugs (>50) exist which can inhibit invadopodia assembly or ECM degradation [101]. Such drugs generally target families of GPCRs, ion channels, receptor and non-receptor tyrosine kinases, phosphatases, cytokines, growth factors and proteases. In addition, cancer cells can be steered away from assembling invadopodia by reducing collagen I cross-linking [48], [102]. While some of these drugs were identified in preclinical trials aimed at invadopodia, many of them are already in clinical trials or available on the market for other applications, and can be re-purposed as invadopodia inhibitors.

It is also important noting that, for future drugs that specifically target invadopodia to be properly validated in the clinic, ideal design of clinical trials may require stepping away from current end points. As of now, Phase II is focused on the shrinkage of the primary tumor or existing metastatic lesions, while the potential metastatic prevention is only considered if the drug is approved and enters the Phase III [103]. However, targeted invadopodia inhibitors may not affect growth of primary tumors or lesions. Depending on the target, primary tumor growth may even increase, if the drug causes the phenotypic switch from invasion to proliferation [104], [105]. Hence, new measurables, such as length of time before a new metastatic lesion is observed, are necessary to properly evaluate benefits of metastasis-preventing drugs [103].

Collectively, these studies suggest that invadopodia may hold a great potential for individual risk assessment as prognostic marker for metastasis or effectiveness of therapy, as well as therapeutic target.

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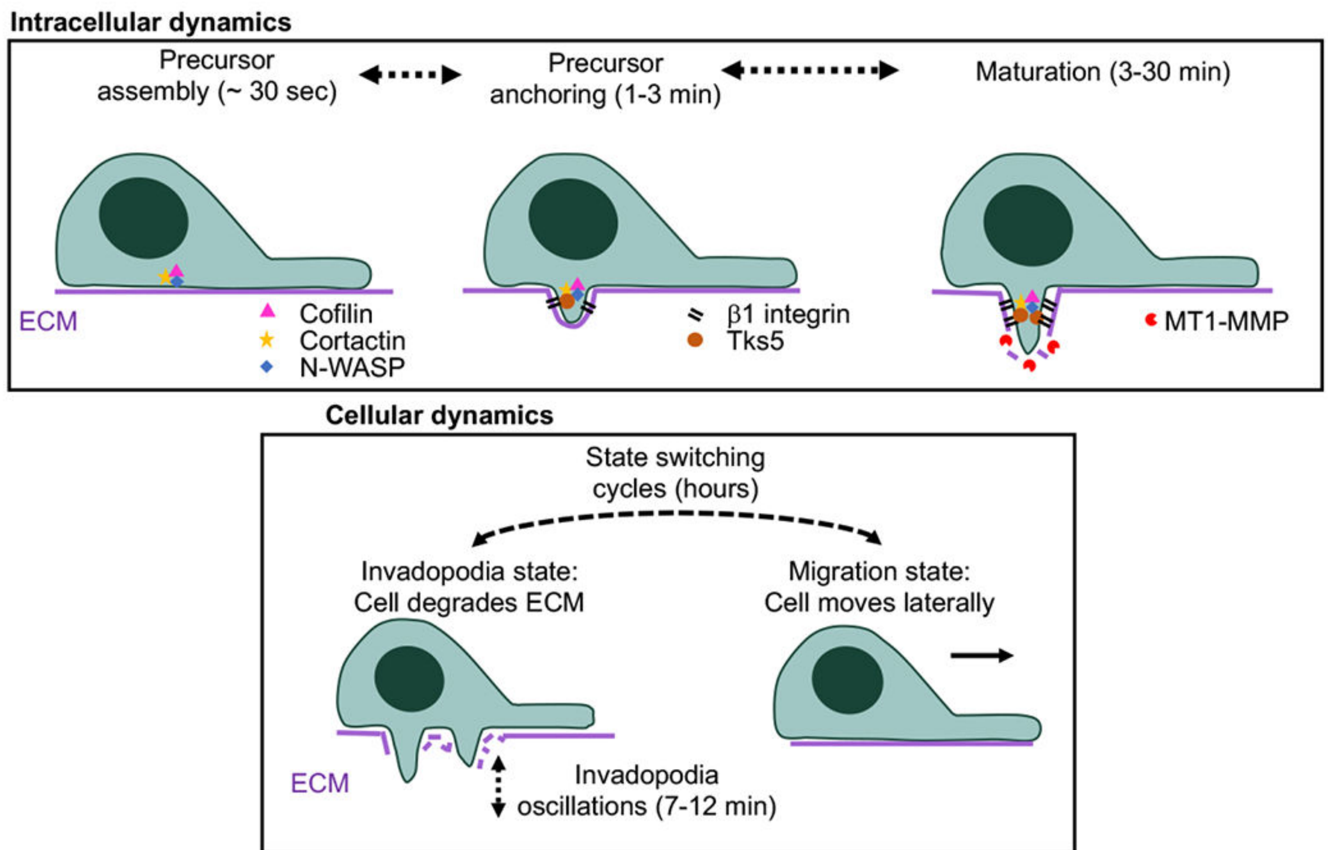

Figure 1. Invadopodia dynamics at different timescales:

Inside the cell, assembly of invadopodium precursor, precursor anchoring to the ECM and recruitment of MT1-MMP leading to invadopodium maturation occur on the timescale of minutes. Once invadopodium starts degrading ECM, cell remains static for several hours in the Invadopodia state. During this time, on the minute timescale, invadopodia oscillate between extension and retraction, controlled by actin polymerization and depolymerization cycles. When degradation of the adjacent ECM results in ECM loosening and accumulation of ECM fragments, cell disassembles invadopodia and enters Migration state, moving laterally towards the new pool of native ECM.

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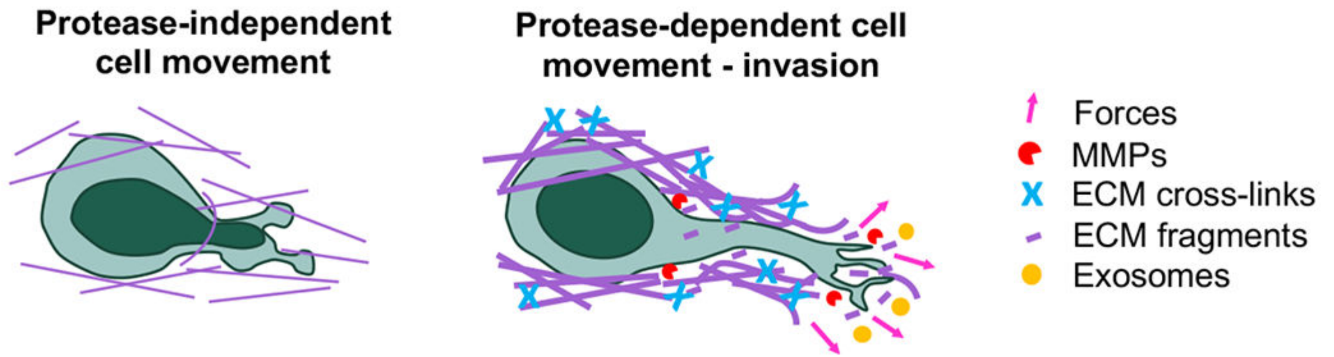

Figure 2. Invadopodia are agents of protease-dependent motility:

In compliant matrices, with pore sizes commonly larger than cell nucleus, cells can squeeze through using amoeboidal, protease-independent motility. In contrast, matrices which are dense, stiff, cross-linked and with pore sizes smaller than nucleus deformability, require assembly of invadopodia to deliver proteases, exosomes and forces to the ECM, resulting in generation of ECM fragments and enlarged pore sizes in ECM.

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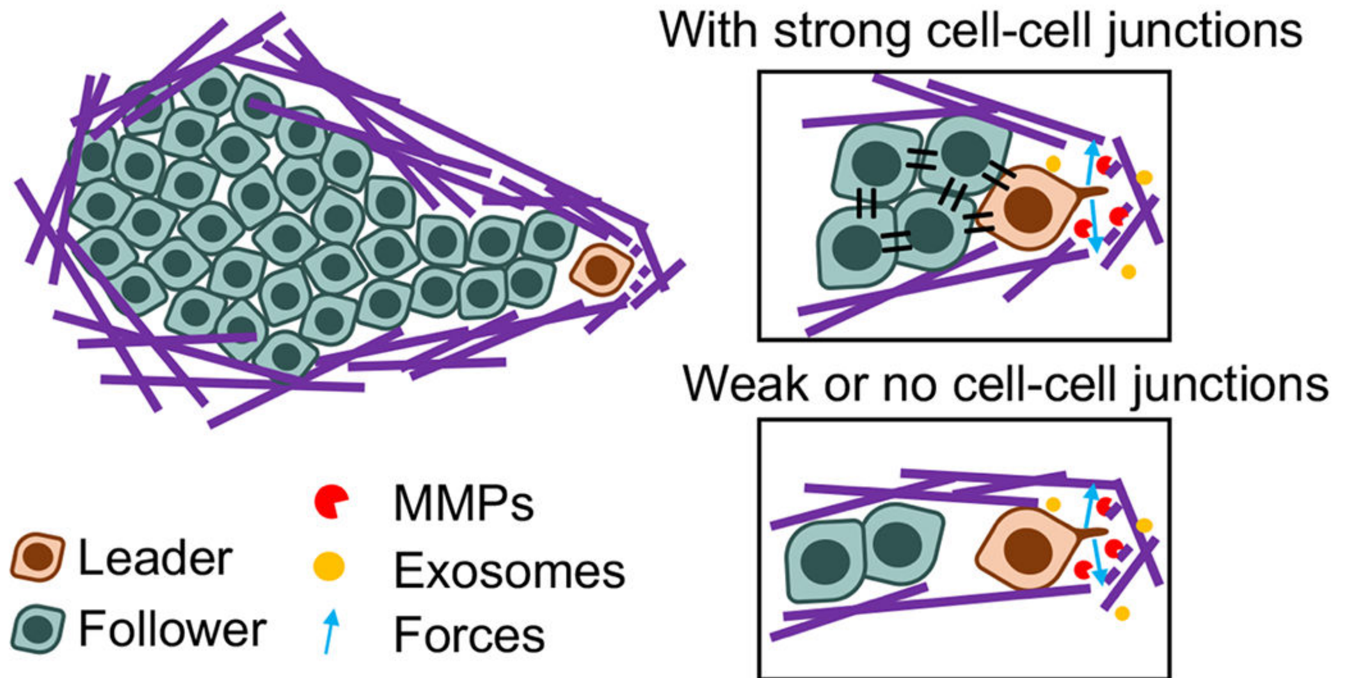

Figure 3. Invadopodia role in collective invasion:

During collective invasion, leader cancer cells form invadopodia that locally degrade the ECM by secreting MMPs. Followers migrate behind them as a cluster (with strong cell-cell adhesions among cells) or as a stream (with weak or no cell-cell adhesions). Invadopodia exert protrusive force onto the ECM, generating mechanical deformations. In addition, invadopodia release MMP-containing exosomes, resulting in long-range ECM degradation.

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Table 1.

Summary of intrinsic and extrinsic factors affecting invadopodia.

Biophysical factors	Stiffness [104, 105]
	ECM crosslinking [27], [106]
	ECM pore size [106], [56]
	ECM plasticity [55], [59]
	ECM Fibrillar topography [107], [108]
Biochemical factors	Growth factors (EGF, PDGF, VEGF, TGFb, HB-EGF, SDF-1a, HGF, CSF1) [18], [109]
	Chemokines [18], [23]
	ECM fragments [110]
	ECM-associated proteins [111]
	Hypoxia [112]–[116]
	pH [43]
	Metabolism (pyruvate, fatty acid synthesis) [119]–[122]
Genetic factors and gene expression	Actin, actin-related proteins and adaptors (cortactin, Tks5, N-WASP, ARP2/3) [18], [20]
	Non-receptor tyrosine kinases (Src [123], Arg [124] and Pyk2 [125])
	Proteases (MT1-MMP, MMP2, MMP9, ADAM12, 15 and 19 [126], [127], serine proteases seprase and DPP4 [31]) [18], [20]
	Adhesion molecules (integrin b1 and b4 [21], talin [128]) [18], [20], [129]
	RhoGTPases and their regulators (Rac1 [130], Cdc42, Rab GDI-1 [131], RalB [132], TOC10 [133]) [134] RhoGAPs (ARHGAP17 [135]) RhoGEFs (Arhgef5 [136], TIAM1 [137])
	Transcription factors (Twistl [138]
Epigenetic factors and cellular state	Cell cycle [48]
	Localized accumulation of mRNA and RNA-binding proteins [139] Non-coding RNAs (circular, microRNAs and long non-coding RNAs) [139], [140]
	Relative cell position in 3D: leader-follower [44]
Cell-cell contacts	Macrophages [25]