Directed cell invasion and migration during metastasis

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Metastasis requires tumor cell dissemination to different organs from the primary tumor. Dissemination is a complex cell motility phenomenon that requires the molecular coordination of the protrusion, chemotaxis, invasion and contractility activities of tumor cells to achieve directed cell migration. Recent studies of the spatial and temporal activities of the small GTPases have begun to elucidate how this coordination is achieved. The direct visualization of the pathways involved in actin polymerization, invasion and directed migration in dissemination competent tumor cells will help identify the molecular basis of dissemination and allow the design and testing of more specific and selective drugs to block metastasis.

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Introduction to cancer metastasis
Metastasis is a multistep process where tumor cells disseminate from the primary tumor and colonize distant organs [1]. Metastasis accounts for more than 90% of all cancer related deaths [2,3]. The steps include tumor cell invasion of basement membranes and the surrounding tissue, intravasation into blood vessels, survival there, extravasation and/or growth at different organ sites. To achieve these steps, precise coordination of cell movement and matrix remodeling are essential [4]. At any given time, only a small proportion of tumor cells are invading and disseminating [5,6]. Understanding the mechanisms that drive motility and invasion of these tumor cells is crucial to better understand metastasis.

Escaping the tumor: protrusions with matrix degradation activity
EMT (epithelial–mesenchymal transition) is believed to be an important early step in the conversion of tumor cells into a migratory population capable of systemic metastasis [7,8]. To become migratory, the first barrier that tumor cells must overcome is their epithelial basement membrane (Figure 1). In order to cross basement membranes, tumor cells are postulated to form invadopodia. There is growing evidence that the formation of invadopodia is part of the EMT process [9,10]. It is unknown the degree to which carcinoma cells must proteolytically digest the epithelial basement membrane as they initially invade and whether stromal cells are also involved in this process. However, there is good evidence for the involvement of invadopodium-mediated proteolysis of vascular basement membranes during intravasation and metastasis [11].

Invadopodia are defined as invasive actin polymerization dependent protrusions found specifically on cancer cells that degrade matrix. They were first described in invasive tumor cells such as the human malignant breast tumor cell MDA-MB-231 [12] and human malignant melanoma cells [13]. In this review we are going to focus on describing invadopodia which, by definition, are only formed by invasive tumor cells and since they have unique characteristics that differentiate them from other matrix degrading organelles described in other cell types [14]. These other degrading organelles are called podosomes (macrophages), rosettes (fibroblasts) and more generally invadosomes (reviewed in [15**]).

When plated on 2D surfaces, tumor cells form invadopodia on their ventral surfaces which degrade extracellular matrix through the delivery of metalloproteases like MT1-MMP [16,17]. Recent studies have shown that the formation of invadopodia is a multistep process involving actin-binding proteins and complex signaling cascades [18]. Initiation of invadopodium assembly involves ‘invadopodium precursor’ formation before maturation to the degradation competent invadopodium. Cortactin phosphorylation by Arg-kinase regulates cortactin binding to other proteins including cofilin and NCK1 and this phosphorylation plays a critical role in regulating actin polymerization and precursor assembly [18–20] (reviewed in [21**]). It has been proposed that at the membrane level, caveolin regulates invadopodium formation through the regulation of membrane cholesterol levels [22], and also that extracellular matrix rigidity can influence the formation of invadopodia [23] suggesting that extra-cytoskeletal factors also regulate their formation.

Invadopodia in chemotaxis
Recent results have demonstrated that invadopodia are involved in directional polarization, signal sensing and directional protrusion during chemotaxis in 2D [24] as
Invadopodia and locomotory protrusions are functionally coupled during both 2D and 3D migration. In 2D invadopodia and pseudopodia (lamellipodia) are separated in space. In 3D both types of protrusions are found together at the cell front. Common signaling and cytoskeletal pathways used by both motility supporting protrusions such as pseudopodia and invasion specific protrusions such as invadopodia indicate a high degree of molecular integration and cross talk between pseudopodia and invadopodia allowing efficient invasion coupled migration in 2D and 3D.

Types of tumor cell motility
For some time it has been generally agreed that tumor cell motility is necessary for tumor cells to metastasize [30]. Once tumor cells have passed through their basement membrane they have to migrate through the extracellular matrix-degrading activity of invadopodia and pseudopodia appears to be a crucial step in the invasion process. A large amount of evidence suggests that matrix-degrading processes such as the activity of the matrix metalloproteinases (MMP) are involved in the degradation of extracellular matrix components such as collagen. MMPs are located in both pseudopodia, and invadopodia and the generation of a matrix-degrading activity seems to be a prerequisite for efficient tumor cell motility. It is likely that the generation of a matrix-degrading activity depends on the activity of these proteases.

Invasion, migration and intravasation in tumors. Tumor cells undergoing EMT form invadopodia and acquire a migratory phenotype. Degradation of basement membranes and extracellular matrix during migration is achieved by invadopodia. Cells migrate on the extracellular matrix (ECM) forming pseudopodia. There is evidence that invadopodia are involved in directional migration and chemotaxis at this step. Once tumor cells reach a blood vessel they are believed to again use invadopodia to degrade the basement membrane and enter the blood stream.

Normalized text:

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These results raise an interesting question: are invadopodia only necessary for degradation, or do they have other functions such as orienting tumor cells toward chemotactic signals such as those associated with blood vessels in vivo? The importance of chemotaxis in tumor and stromal cell migration in vivo and in tumor cell dissemination and metastasis has been reviewed recently in detail [21**]. An important insight to come from these considerations is that invadopodia are involved in chemotaxis during migration in both 2 and 3D (Figure 2). Previous reports of human tumor cells migrating in three-dimensional matrices indicate that matrix-degrading invadopodia localize to the leading edge of the invading cell where the secretory machinery and metalloproteases are located [26]. Recently, Magalhaes et al. [27] were able to identify invadopodia during cell invasion in 3D matrix. MDA-MB-231 cells in a 3D matrix contain cortactin, Tks5, MT1-MMP and matrix degradation (which together in tumor cells are unique markers of invadopodia) localized in protrusive structures at the leading front of the cell in dense native matrix consistent with studies of 3D cell migration by breast carcinoma cells [28]. Similar results implicating invadopodium-mediated proteolysis at the cell front during invasion and migration in vivo in mammary tumors have been observed [11]. Wolf et al. [29] have reported that tumor cells invading 3D pepsin digested collagen also exhibit pericellular degradation to widen the hole available for pseudopodium-mediated migration but did not observe proteolysis at the cell front in this pepsin treated collagen. Taken together, these 3D and in vivo studies indicate that proteolytic activity at the cell front is necessary for tumor cell invasion and migration in 3D and that this requirement is relaxed in pepsin treated collagens lacking telopeptides and cross-linking. Furthermore, common signaling and cytoskeletal pathways used by both locomotory protrusions, such as pseudopodia, and invasive protrusions, that is invadopodia, indicate a high degree of molecular integration and cross talk between pseudopodia and invadopodia allowing efficient invasion coupled migration in both 2 and 3D (Figures 1 and 2) [21**].
matrix for long distances to disseminate efficiently via blood vessels and lymphatics (Figure 1). But, how do tumor cells move long distances in the tumor? The development of novel imaging techniques has illuminated these processes [31–33]. High resolution multiphoton imaging of tumors in vivo has shown that tumor cells use both collective and single cell motility (reviewed in references [21**,34]). Breast carcinoma cell motility is characterized by solitary amoeboid movement. Cells undergoing amoeboid movement in vivo move at high speeds (~4 μm/min) compared to other types of cell motility described in vivo [31].

An important question is: why do tumor cells display these different types of motility? A common feature of cell motility in vivo is the formation of F-actin rich protrusions which cells use to extend forward to adhere to their surroundings, followed by contraction of the trailing end. These coordinated events, called the motility cycle, propel the cell forward. Coupled to these events is the formation of invasive protrusions used to penetrate through the extracellular matrix and to overcome matrix barriers to migration. Historically, protrusions at the leading edge of motile cells were named based on their shape: filopodia (needle shape), pseudopodia (round), lobopodia (cylindrical) and lamellipodia (flat veils) [35]. Lamellipodia are observed when cells are plated on 2D substrates. All are F-actin rich and actin polymerization is required for their protrusion [36,37]. Invasive tumor cells form pseudopodia in vivo (functionally equivalent to lamellipodia but three dimensional [38]) in response to the EGF secreted by the tumor associated macrophages, as part of the tumor cell/macrophage paracrine loop described in breast tumors [21**,39]. F-actin rich pseudopodia are the defining morphological feature of fast moving amoeboid cells and are involved during chemotaxis to direct tumor cells toward blood vessels before intravasation [11,31] (Figure 1). Different reports have described the importance of amoeboid motility for cell migration in vivo [40–42]. This work identifies ROCK, and its regulator PDK1, as important mediators of cortical actomyosin contractility part of the motility cycle [41,42], and Rac, as a central GTPase involved in the interconvertability of different modes of tumor cell motility [40,43].

The formation of these distinct protrusive structures during the cell motility cycle of tumor cells during migration and dissemination in vivo highlights the fact that tumor cells adapt to different types of matrix composition, as proposed for other cell types [44]. We hypothesize that the tumor microenvironment is responsible for these different motility behaviors. The interactions with different stromal cell types, different matrix compositions and different chemoattractants further determine how tumor cells behave within, and escape from, the primary tumor. For example, the formation of invasive protrusions (invadopodia) and locomotory protrusions (pseudopodia) is regulated by stromal cells in the tumor microenvironment including fibroblasts (squamous cell carcinoma) [45] and macrophages (breast carcinoma) [21**].

**Regulation of actin polymerization and metastasis**

The formation of long, force-supporting membrane protrusions requires actin polymerization. Signaling pathways are altered in invasive tumor cells to increase their actin polymerization activity and motility [5,6]. The Rho-family GTPases have been directly linked to motility and protrusion formation through their ability to activate the signaling targets that direct upstream actin cytoskeleton modifying proteins. Among the 20 members of the family, the most studied have been RhoA, Rac1 and Cdc42. The close isoforms of Rho including RhoA, RhoC and RhoB, play different roles in cancer [46–49]. RhoC expression is upregulated in metastatic tumor cells isolated from lung metastases [50] and invasive human breast tumor cells [48**]. Rac1 plays a critical role in metastasis regulating the spatial formation of invadopodia to tightly focus the matrix degradation [48**]. However, RhoA seems to play a different role in invasion, regulating MMP trafficking and Rac1 activation [47–49]. Effectors of GTPases, like the formins; and the Arp2/3 complex, have also been shown to be involved in metastasis through their roles in motility and protrusion formation (reviewed in [51]).

The coflin activity cycle is regulated by GTPases through ROCK [52]. Coflin plays a critical role in promoting protrusions through the formation of free actin barbed ends needed for new actin polymerization. The coflin pathway is upregulated both at the level of gene expression and protein activity [5,53] in metastatic tumor cells where the coflin activity cycle is involved in actin polymerization, protrusion formation, chemotaxis, motility and invasion [48**,54]. To explain the activation of coflin locally, a Local Excitation Global Inhibition (LEGI) model has been proposed. Global inhibition of coflin activity through coflin phosphorylation mediated by RhoC, restricts coflin activity just at the very tip of a motility supporting protrusion or within the core of an invadopodium [48**,53] suggesting how these two types of protrusions might be coordinated at the cell front.

Rac1 GTPase, a member of the Rac GTPase subfamily, is necessary for the formation of lamellipodia. It has been proposed that the anti-capping protein Mena is involved in acting as a negative regulator of Rac1 activity [55]. In addition, it has been shown that the splicing patterns of Mena isoforms change in invasive versus non invasive tumor cells in vivo. Invasive tumor cells upregulate MENAINV and downregulate another Mena isoform, MENA 11a [56]. Overexpression of MENAINV promotes invadopodium formation and matrix degradation and also
lamellipodium formation contributing to tumor cell invasion, dissemination and metastasis [57–60].

**RhoGTPases and invadopodia: spatiotemporal RhoC GTPase signaling**

Different GTPases have been implicated in the formation and function of invadopodia including Cdc42, RhoA and Rac1 [17,61,62]. However, recent reports show that GTPases display spatiotemporal activation dynamics important for the precise control of different signaling pathways [63*]. Different regulators including GEFs, GAPs and GDIs regulate the GTPase activation cycle. To decipher such complex and dynamic activation kinetics, traditional approaches including biochemical strategies or simply tagging these molecules with fluorescent proteins, fall short in detecting these phenomena in living cells. Fluorescent biosensors for the detection of Rho GTPase activation have emerged as critical and powerful tools allowing direct interrogation of GTPase activation at subcellular resolution and in the time scale of seconds [64–66] (Figure 3). These fluorescent biosensors based on the fluorescence resonance energy transfer (FRET) rely on the conformational reorientation within the engineered molecule upon ‘activation’ of the built-in GTPase through the guanine nucleotide exchange. This event causes the shift in the dipole–dipole coupling angle between the two fluorescent proteins within the biosensor to affect FRET; thus, through monitoring the ratio of the FRET to donor fluorescent emissions, one can determine the relative protein activation states within a single living cell.

Using a FRET-based RhoC biosensor, a novel pathway controlling spatial restriction of invadopodium activity has been described (Figure 4). RhoC is activated surrounding the invadopodium center generating a ring pattern achieved by the spatial placement of p190RhoGAP inside the center, and p190RhoGEF outside the center. The combination of a specific GEF and GAP in these locations tightly regulates and focuses the region of RhoC activation (Figure 4). As a consequence, the activation of the RhoC/ROCK/LIMK pathway results in cofilin phosphorylation in the outer ring thereby focusing the cofilin activity to generate barbed ends and actin polymerization within the center, focusing the matrix degradation activity for efficient invasion [48*]. These results show that controlling the RhoGTPase signaling is not only achieved by the quantity of GEFs and GAPs acting on a specific GTPase, but also by the spatial and temporal placement of these components that will specifically target the activity of the GTPase and its signaling pathway.

**Concluding remarks and future directions**

To date, drugs capable of blocking metastasis are not available. At least two reasons for this are (1) that such a complex process is still not well understood and (2) there are no reliable end point markers for evaluating drug performance in blocking metastasis. Invadopodia are unique protrusions with matrix degrading activity formed specifically by invasive tumor cells that have an essential role in invasion, migration and chemotaxis during metastasis, making them an attractive functional target to interfere with metastasis and as a potential marker for assessing drug performance. Defining the molecular features of invadopodia under physiological conditions will help in the design of specific drugs that could block...
metastasis in the future. Proteins involved in the formation of invadopodia are upregulated in the invasive and migrating population of tumor cells. The signaling pathways involved in invadopodium formation may not be present in normal cells. Understanding these signaling pathways will clarify the molecular basis of metastasis and allow the design and testing of more specific and selective drugs to block metastasis.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:
- of special interest
- of outstanding interest


This paper shows that TWIST1, a central regulator of EMT, promotes invadopodia formation.


A comprehensive review on the different degrading structures found in a variety of cell types.


A comprehensive review about how chemotaxis is essential to cancer progression.


This paper shows, for the first time, RhoC activation at invadopodia. It describes how RhoC activity is spatiotemporally regulated by p190Rho-GEF/p190RhoGAP.


First paper identifying RhoC as a gene upregulated in metastatic tumor cells.


This paper describes the first design of the RhoA single chain biosensor that retains its original C-terminus permitting the interaction with endogenous GDI.


67. Kurokawa K, Matsuda M: Localized RhoA activation as a requirement for the induction of membrane ruffling. Mol Biol Cell 2005, 16:4294-4303. This paper is the first description of a single chain biosensor for RhoA. In this design the Rho GTPase and the small binding domain are placed within the internal portion of the single-chain biosensor construct, not permitting the interaction with endogenous GDI.