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Trespassing cancer cells: ‘fingerprinting’ invasive protrusions reveals metastatic culprits

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Abstract

Metastatic cancer cells produce invasive membrane protrusions called invadopodia and pseudopodia, which play a central role in driving cancer cell dissemination in the body. Malignant cells use these structures to attach to and degrade extracellular matrix proteins, generate force for cell locomotion, and to penetrate the vasculature. Recent work using unique subcellular fractionation methodologies combined with spatial genomic, proteomic, and phosphoproteomic profiling has provided insight into the invadopodium and pseudopodium signaling networks that control the protrusion of invasive membranes. Here I highlight how these powerful spatial “omics” approaches reveal important signatures of metastatic cancer cells and possible new therapeutic targets aimed at treating metastatic disease.

Introduction

When cancer cells acquire the ability to invade tissues and metastasize throughout the body, the likelihood of disease recurrence is significantly increased and patient prognosis for survival is greatly diminished [1,2]. In fact, the majority of patients (>90%) succumb to cancer due to systemic cell metastases. Currently, there are no effective treatments available to selectively combat these invasive cells. The development of therapeutics and treatment strategies has been hindered by the inherent difficulty in studying the complex and dynamic processes of cell metastasis in vivo and the notable lack of specific biomarkers that reveal the exact nature and location of malignant cells [1]. Therefore, unique biomarkers that serve as specific metastatic ‘fingerprints’ are needed for the design of sensitive detection methods and for the development of specific therapeutics [3]. Invadopodia and pseudopodia are specialized membrane protrusions that facilitate the dissemination of metastatic cancer cells (Figure 1A) [4,5]. In this review, I discuss how studying these structures may hold promise to the identification of specific metastatic signatures and drugable targets designed to treat metastatic cancer.

Invadopodia and pseudopodia formation drive cancer metastasis

Cancer cell metastasis is a complex cascade of multiple biological processes that culminates in the colonization of secondary organs and tissues of the body [1]. One of the distinguishing hallmarks of metastatic cells that disseminate from solid tumors is their ability to degrade the basement membrane and invade into the surrounding tissue

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parenchyma [1]. Morphometric and biochemical analyses have shown that invasive cells localize proteases to actin-rich invadopodia. Cancer cells use invadopodia to attach to and degrade extracellular matrix (ECM) proteins, which constitutes the basement membrane (figure 1B) [6,7]. Invadopodia are characterized by cell-matrix contacts, which are highly enriched with filamentous actin (F-actin) bundles oriented perpendicular to the substrate [8–10]. This structure is surrounded by integrin adhesion receptors and radial actin fibers, which are regulated by multiple signaling pathways involved in actin polymerization, membrane trafficking, and protein phosphorylation. Although the process is poorly understood, invadopodia transform into larger membrane protrusions called pseudopodia (figure 1A) [11,12]. The transition from invadopodia to pseudopodia (lamellipodia) is a key event that initiates cell body propulsion through the basement membrane and into the surrounding tissue and stroma. The physical process of cell translocation is controlled by actin-mediated protrusion of a leading pseudopodium at the cell front (which pulls the cell forward) followed by tail retraction at the cell's rear compartment. The basal localization of the basement membrane as well as ECM proteins and growth factors present in the extracellular environment can provide instructive cues to invading cancer cells. It is believed that these factors work along with genetic abnormalities of the cancer cell to breach the basement membrane and drive a polarized invasive process [1,13,14].

After entering the extracellular environment, current evidence indicates that invading cells randomly locate or are guided to the vascular system using poorly defined mechanisms [15,16]. Here again these cells use their proteolytic and protrusive machineries to penetrate the basement membrane of the vessel wall and translocate through the endothelium, gaining access to the blood stream (Figure 1c) [17,18]. The cells are then transported by normal circulation to secondary organs where they passively lodge or attach to the endothelium using specific adhesion mechanisms. Once again these cells invade the endothelial barrier and extracellular tissues to establish secondary tumors [17,18]. Thus, the ability of cancer cells to form invasive membrane protrusions is a key factor in the metastatic cascade driving the dissemination of cancer cells in the body. As discussed below, recent work indicates that mining the components of these specialized structures may hold the key to the identification of unique biomarkers and therapeutic targets directed at eradicating metastatic cancers.

Finding the needle in the haystack: mining the invadopodiome for metastatic signatures and drugable targets

The majority of studies have used immunofluorescent and fluorescent protein tagged imaging technologies to localize specific proteins to invadopodia [6,8,9,19]. Over the years many important invadopodia proteins have been defined and functionally studied in this manner. More recently, the purification of invadopodia from cancer cells combined with proteomic methods have led to the identification of a significant number of new invadopodia-associated proteins [20]. While the molecular components of the invadopodiome are still being defined, this collective body of work has revealed important proteins that could be evaluated as a means to detect and battle metastatic cancer cells in the clinical setting. Table 1 shows a partial list of the proteins identified in the invadopodiome. Of these proteins, enzymes and their specific substrates are attractive candidates to target metastatic cells, as they have been traditionally drugable by the pharmaceutical industry. For example, src kinase and its known substrates Tks4/5, paxillin, and cortactin have been shown to play a key role in invadopodia regulation in cancer cells [4,5,19,21,22]. While the causative link needs to be established, these proteins show aberrant expression and activity in various human cancer cells and they correlate with metastasis and poor patient survival [23,24]. Several important pharmacological agents have been designed to inhibit the catalytic activities of src family kinases [25]. These agents are under intense scrutiny in the clinic for efficacy against several cancers including breast, prostate, melanoma, and

colorectal cancer with promising results. It is possible that the increased survival seen in some patients treated with src inhibitors is, at least in part, due to inhibition of invadopodia formation and reduced metastatic load. Also, antibodies and phosphospecific antibodies to src substrates like Tks5 and cortactin could be used as prognostic indicators of disease progression and patient outcome as well as biomarkers that predict sensitivity to src therapeutics in different cohorts of cancer patients.

Src is not the only low hanging fruit waiting to be picked from the invadopodiome. Other enzymes can regulate invadopodia in cancer cells (e.g. Fak, Abl family kinases, PKC, metalloproteases) (table 1). In fact, small molecule inhibitors to some of these enzymes are already being scrutinized by the pharmaceutical industry as a means to improve survival of cancer patients [26,27]. Finally, the identification of novel invadopodia-associated proteins with no reported function may be the Holy Grail for metastatic cancers. While these proteins are uncharacterized, powerful computational informatics programs that identify domain structures, consensus phosphorylation signatures, kinase networks, and genome structure can be used to tentatively assign proteins to signaling pathways and to predict cell and disease functions [28,29]. Functional studies in cells and animal models of cancer can then be designed to determine the precise role of these novel genes and proteins in invasive cancer cells. A general overview of the “omics” strategies being used to mine the invadopodiome and pseudopodiome for metastatic markers are shown in figure 2.

Mining the pseudopodiome for metastatic signatures and druggable targets

Several laboratories have been mining the pseudopodiome for markers of invasive and metastatic cells with success [30–33]. These studies have capitalized on the ability to selectively isolate the pseudopodium from the cell body using 1–3 μm microporous filters for mass spectrometry-based analyses (Figure 3) [31,34–37]. This technology has been described in detail and is shown schematically in figure 3. This model system recapitulates physiological events associated with cancer cell metastasis including pseudopodium invasion through small openings in the ECM and vessel wall (Figure 1C). Collectively, these studies have revealed thousands of pseudopodia-associated proteins and their signaling networks. Some of the key proteins identified in the pseudopodium are shown in table 1. Using this fractionation technique and quantitative proteomics, a recent study determined the relative distribution of 3509 proteins and 228 distinct sites of phosphorylation in the pseudopodium and cell body compartments [30,38]. This spatial proteomics approach combined with informatics and network analyses revealed that major pseudopodial networks control integrin signaling, cytoskeleton remodeling, and axon guidance mechanisms, whereas, the cell body proteome largely consists of DNA/RNA metabolism, cell cycle regulation, and other basic housekeeping functions.

While the comprehensive profiling of the different subcellular compartments provided insight into the spatial organization of protein networks in migratory cells, it also revealed many new pseudopodium-associated proteins that have not been previously linked to cell migration or are of unknown function altogether. These proteins represent possible new signatures of invasive and metastatic cells. For example, this approach revealed a novel and previously uncharacterized non-receptor tyrosine kinase, called PEAK1 (pseudopodium-enriched atypical kinase one) [38,39]. PEAK1 is amplified in multiple human malignancies including metastatic pancreatic ductal adenocarcinomas (PDAC), where it correlates with poor patient survival. PEAK1 overexpression in human PDAC cells enhances pseudopodia formation, cell migration, cell proliferation, tumor formation, and metastasis in animal models of cancer [40]. In contrast, knocking down PEAK1 expression in human PDAC cells diminishes these responses. The fact that PEAK1 critically regulates cell proliferation and metastasis, and is a catalytically active tyrosine kinase, makes it an attractive target for

development of a small molecule kinase inhibitor aimed at treating primary and secondary tumor growth [39]. Although it has not been tested, PEAK1 may also play a role in invadopodia formation as it is phosphorylated by src and can modulate src kinase activity in cancer cells [40]. The discovery of PEAK1 kinase exemplifies the power of using subcellular fractionation and spatial proteomics to reveal novel, and potentially druggable proteins, that mediate cancer cell metastasis.

Pseudopodia purification combined with genomic and proteomic profiling has also revealed an important link between the ability to form pseudopodia protrusions and epithelial to mesenchymal transition (EMT), which is seen in many metastatic cancer cells [33]. EMT is the transformation of epithelial cells toward a highly invasive mesenchymal state characterized by the loss of cell-cell junctions and cell surface E-cadherin [1,41]. These cells also acquire a fibroblastic-like morphology with numerous actin-rich pseudopodia. The EMT transformation is believed to arm cells with the ability break away from the advancing front of the primary tumor, switch from collective to individual cell migration, and to invade through the ECM-rich stroma into the vasculature. The genetic and proteomic profiling of pseudopodia from multiple metastatic human cancer cell lines from various origins led to the identification of 19 common pseudopod-specific proteins [33]. Interestingly, functional knockdown of four of the pseudopod signature proteins (AHNAK, septin-9, eIF-4E, S100A11) reversed the EMT phenotype inducing a mesenchymal to epithelial transition (MET). The MET transition seen in these cells was associated with reduced F-actin content, actin turnover rate, and pseudopodium protrusion indicating that they work through a common mechanism involving the remodeling of the actin cytoskeleton. Although the precise mechanism is not yet understood, these findings are important because they demonstrate for the first time that the actin dynamics of the pseudopodium are critically involved in controlling EMT transition and, thus, the invasiveness of metastatic cancer cells. This work is also important because it is the first attempt to define a common set of invasive signatures associated with pseudopodia formation and cancer cell metastasis. Indeed, some of the identified signatures already show a high degree of cancer relevance including eIF4E, septin-9, and S100A11 [42–46]. Finally, in some cancers an EMT phenotype has been associated with cancer stem cell-like properties [1,2]. This suggests the possibility that the pseudopodial cytoskeleton may also regulate cancer stem cell differentiation by virtue of its ability to relockade of pseudopodial protein functions therapeutically could reverse the invasive EMT phenotype and induce cancer stem cell differentiation, which could be exploited to benefit cancer patients.

Mining pseudopodium-enriched RNAs for metastatic signatures

Recent elegant studies showed that mRNA transcripts and protein translation are spatially localized in membrane protrusions of motile cells, which are regulated by RhoA and adenomatous polyposis coli tumor suppressor protein [32,34,46–50]. Using the pseudopod and cell body fractionation scheme combined with microarray technology, global analysis of the pseudopodium transcriptome revealed more than 260 genes enriched in the pseudopodium domain, providing a rich source of potential signatures of motile and invasive cancer cells. Interestingly, comparison of the pseudopodial transcriptome and proteome revealed that many of the cytoskeletal, focal adhesion, glycolytic enzymes, and chaperones detected in the proteome were not represented in the transcriptome. Instead, the majority of pseudopodial transcripts are involved in mRNA transport, protein translation, and signal transduction. Although the transcriptome has been examined in invadopodia, many components of the protein translation machinery are enriched in the invadopodium [20]. Furthermore, quantitative proteomics of the integrin adhesome show that the RNA translation machinery is enriched at sites of cell-matrix contact [51]. This important work suggests that integrin receptors may play a role in recruiting and regulating RNA translation

in the invadopodium. Collectively, these data indicate that the translation machinery is highly enriched in invasive membrane protrusions and integrin adhesions, which places them in prime position to regulate cancer cell metastasis. However, additional work is necessary to determine how RNAs localize to the invadopodium and to what extent the levels of specific mRNAs and their protein products are expressed in invasive protrusions from metastatic cancer cells. It would also be interesting to determine if small regulatory microRNAs are spatially polarized in migrating cancer cells and whether this contributes to the metastatic process.

The fact that the protein translation machinery is highly enriched in dynamic membrane protrusions has important therapeutic implications for treating metastatic cancers. Inhibition of translation is considered a promising new area for the development of novel anticancer agents [45,46,52]. Many hyper-proliferative cancers deregulate translation components such as the eIF4F complex (eIF4E, eIF4G, and eIF4A) and mTOR signaling, which regulates the rate limiting translation initiation step of protein synthesis. Similarly, the eukaryotic translation initiation factor 5A (EIF5A) is enriched in the pseudopodium and is also amplified in several human cancers [53,54]. It is also under investigation as a potential target for anticancer therapy. While these factors are believed to augment increased oncogene expression and thus excess cell proliferation, they could also lead to hyper production of membrane protrusions and the activation of the metastatic machinery. In fact, several compounds have already been identified that show promise as translation inhibitors with anticancer properties including hippuristanol, rapamycin, metformin, and ciclopirox olamine [45,46]. It would be interesting to determine if these drugs can also block invadopodia and/or pseudopodia formation, EMT and cancer metastasis.

Conclusion

The majority of patients succumb to cancer because they already have metastases at the time of diagnosis. So the critical clinical question is how to identify and therapeutically target established metastases. Only then will we be able to truly eradicate cancer. This is a difficult problem to address because metastasis involves a complex cascade of events and metastatic cells are commonly refractory to current chemotherapies. However, the fact that highly invasive cancer cells are endowed with aberrant abilities to produce membrane protrusions may very well be exploitable as a unique means to identify and inhibit specific molecular features of established metastases. The ability to directly purify invasive protrusions combined with rapidly evolving genomic, proteomic, and informatics methodologies has opened the door for deeper and more comprehensive transcriptome and proteome mining strategies. These spatial “omics” approaches will certainly aid in the identification of unique ‘fingerprints’ of metastatic cells. Although still in its infancy, initial profiling work has provided a glimpse into the nature of invasive protrusions and revealed metastatic cell signatures, some of which are possible drugable targets. From these studies, it can be envisioned that future efforts in mining and network analyses of the invadopodiome and pseudopodiome will provide a comprehensive understanding of the molecular signaling mechanisms that control cancer cell invasion and metastasis. This level of understanding together with high content drug screening efforts may lead to the first small molecule inhibitor designed to target metastatic cancer cells. In fact, the first drug screen to identify small molecule inhibitors of invadopodia formation was recently performed with success, revealing several important candidates, which pointed to cyclin-dependent kinase five as a key target [55].

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Figure 1.

Invasive cell protrusions degrade basement membranes and extracellular matrix proteins as well as mediate penetration of the vascular wall, (a) Schematic showing the steps of cancer cell invasion into the extracellular environment. Step1, normal healthy tissue architecture with an intact basement membrane. Step2, Genetic mutations drive cancer cell transformation, proliferation (pink cells), and loss of cell-cell contacts. Genetic mutations in cancer cells in association with changes in the tumor microenvironment drive formation of specialized actin-rich membrane protrusions, called invadopodia, which degrades the basement membrane (red cell). Step 3. Invadopodia transform into larger actin-rich structures called pseudopodia that degrade the surrounding extracellular matrix of the tissue. Step 4. These specialized membrane protrusions provide propulsive forces and a steering mechanism as motile cancer cells degrade and navigate through complex tissues, (b) SCC61 cells (head-and neck cancer) were cultured overnight on FITC-gelatin. Actin-rich

invadopodia puncta were detected with rodamine-labeled phalloidin and fluorescence microscopy. The areas of invadopodia activity (gelatin degradation) are shown as black areas within the green gelatin background. The cell nucleus is shown in blue. Scale bar = 15 μm . (c) Upper schematic depicts invasive cancer cells entering the circulation using invadopodia and pseudopodia protrusions to penetrate through the vascular wall and endothelial barriers. Lower left, confocal image of MDA-MD-435 human cancer cells (red) invading the vessel wall (green) (arrows) in live *Tg[flil:EGFP]* zebrafish. Lower right, computer generated 3-dimensional reconstruction of the boxed area in the lower left image showing a tumor cell protrusion (red) in the vessel lumen (green). Scale bars, left = 20 μm , right = 10 μm .

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Figure 2.

Flow diagram showing how spatial “omics” technologies can be used to mine the invadopodiome and pseudopodiome for metastatic signatures, which could be used for biomarker and pharmaceutical development. MS =mass spectrometry.

Figure 3.

Pseudopodia purification using microporous membranes, (a) schematic illustration pseudopodia purification. Cells are allowed to attach to the surface of the upper chamber containing a 3.0 μm membrane filter. Extracellular matrix proteins or chemokines are placed in the lower chamber to create a diffusion gradient. The cells sense the gradient and extend membrane protrusions through the small openings to the lower surface in the direction of the gradient. The membrane protrusions can then be amputated from the cell bodies which remain on the upper membrane surface. The isolated pseudopodia can then be profiled for RNA and protein components using gene array and proteomic technologies, (b) Confocal image of a GFP labeled cell protruding pseudopodia through the microporous filter (dashed

line), (c) The pseudopodium protruding through the 3.0 μm pore (arrow) was fixed and stained with rhodamine phalloidin to visualize the F-actin cytoskeleton.

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Table 1

*Key proteins that regulate invadopodia and/or pseudopodia formation

Protein	Invadopodia	Pseudopodia	Functional Classification	References
Src	YES	YES	Tyrosine kinase	[4,5,19–22]
FAK/Pyk2	YES	YES	Tyrosine kinase	[6,8,10]
PKC	YES	YES	Serine/Threonine kinase	[6,8,10]
Cortactin	YES	YES	Focal adhesion and actin Cytoskeleton regulation	[4–6,8,10,19–22]
Paxillin	YES	YES	Focal adhesion and actin cytoskeleton regulation	[6,8,10]
$\beta 1$ integrins	YES	YES	Cell adhesion	[8]
$\beta 3$ integrins	YES	YES	Cell adhesion	[8]
Caveolin-1	YES	YES	Membrane signaling	[6,8,10]
Actin	YES	YES	Cell structure/Signaling scaffold	[4–6,8,10,19–22]
Tks4/5	YES	ND**	Adaptor protein and Src substrate	[4,5,22]
p190RhoGAP	YES	YES	GTPase activating protein	[6,8,10]
G protein β subunit	YES	ND	Membrane signal transduction	[6,8,10]
Dynamin-2	YES	YES	Membrane signal transduction	[6,8,10]
CD44	YES	YES	Cell adhesion	[8]
Mena	YES	YES	Cytoskeleton regulation	[8]
MT1-MMP	YES	YES	Extracellular matrix degradation	[6,8,10]
Rac1	YES	YES	Focal adhesion and cytoskeleton regulation	[6,8,10]
Cdc42	YES	YES	Cytoskeleton regulation	[6,8,10]
RhoA	YES	YES	Cytoskeleton regulation and cell contraction	[6,8,10]
N-WASP	YES	YES	Cytoskeleton regulation	[6,8,10,12]
Arg	YES	YES	Abl family tyrosine kinase	[8]
Fascin	YES	YES	Cytoskeleton regulation	[8,12]
AFAP-110	YES	YES	Cytoskeleton regulation	[8]
ARF6/ARNO	YES	YES	Vesicular trafficking	[8]
RhoU/Wrch-1	YES	ND	Focal adhesion and cytoskeleton regulation	[6,8,10,12]
Caldesmon	YES	YES	Cytoskeleton regulation	[8]
Calpain	YES	YES	Cysteine protease	[8]
Palladin	YES	YES	Cytoskeleton regulation	[6,8,10]
Talin	YES	YES	Focal adhesion and cytoskeleton regulation	[6,8,10]
PEAK1	ND	YES	Focal adhesion and cytoskeleton regulation	[38–40]
α -actinin	YES	YES	Focal adhesion and cytoskeleton regulation	[6,8,10,12]
APC	ND	YES	mRNA localization	[48]
LASP-1	ND	YES	Focal adhesion and cytoskeleton regulation	[31]
AHNAK	ND	YES	Membrane cytoskeleton <i>g</i> regulation	[32,33,47]
Septin-9	ND	YES	Tumor suppressor	[32,33,47]
eIF-4E	ND	YES	mRNA/protein translation	[32,33,47]
S100A11	ND	YES	Calcium binding, signal transduction	[32,33,47]
PKA	ND	YES	Serine/Threonine kinase	[56]

Protein	Invadopodium	Pseudopodium	Functional Classification	References
ERK1/2	YES	YES	Serine/Threonine kinase	[8,57-59]
ACLY	ND	YES	Glycolysis regulation	[60]
PAR-2	ND	YES	Receptor signal transduction	[58,59]
β -arrestin	ND	YES	Scaffold protein, signal transduction	[58,59]
EphA2	ND	YES	Receptor tyrosine kinase	[30]
EGF receptor	ND	YES	Receptor tyrosine kinase	[30]
Protein kinase B/Akt	ND	YES	Serine/threonine kinase	[30]

* The complete datasets of mRNAs and proteins identified in the invadopodium and pseudopodium using genomic and proteomic profiling can be found at [20,30,32,33,48]

** Not determined

FAK, focal adhesion kinase. PKC, protein kinase C. N-WASP, Neural Wiskott-Aldrich syndrome protein. Arg, Abelson-related gene. AFAP-110, actin filament-associated protein 110. ARF6, ADP-ribosylation factor 6. PEA1, pseudopodium-associated atypical kinase one. APC, adenomatous polyposis coli. LASP-1, lim and SH3 domain protein one. AHNK, neuroblast differentiation-associated protein. eIF-4E, eukaryotic translation initiation factor 4E. PKA, protein kinase A. ERK, extracellular regulated kinase on and two. ACLY, ATP citrate lyase. Par-2, protease-activated receptor two. EphA2, Ephrin type-A receptor two. EGF, epidermal growth factor.