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Review

Invadopodia: At the cutting edge of tumour invasion

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Abstract

Invasion of tissues by malignant tumours is facilitated by tumour cell migration and degradation of extracellular matrix (ECM) barriers. Several invasive neoplasms, including head and neck squamous cell carcinoma, breast carcinoma, melanoma and glioma, contain tumour cells that can form actin-rich protrusions with ECM proteolytic activity called invadopodia. These dynamic organelle-like structures adhere to, and digest, collagens, laminins and fibronectin. Invadopodia are dependent on multiple transmembrane, cytoplasmic and secreted proteins engaged in cell adhesion, signal transduction, actin assembly, membrane regulation and ECM proteolysis. Strategies aimed at disrupting invadopodia could form the basis of novel anti-invasive therapies for treating patients. Here we review the molecular basis of invadopodia formation with particular emphasis on the intracellular signaling networks that are essential for invadopodia activity and examine the potential role of these structures in glioma invasion.

Keywords: Invadopodia; Glioma; Invasion; Podosome; Extracellular matrix

1. Introduction

Gliomas are the most prevalent malignant brain tumour in adults, causing over 1% of cancer-related deaths. Although there are many histological subtypes as classified by the World Health Organization,[1] gliomas are typically characterized by their infiltrative nature. Treatment for high-grade tumours such as glioblastoma multiforme (GBM) and anaplastic astrocytoma usually includes surgery to debulk the tumour and post-operative adjuvant therapies. The "Stupp protocol" of radiation therapy and concurrent chemotherapy with temozolomide for GBM is associated with a median patient survival of 14.6 months, with 26.5% of individuals surviving for 2 years or longer.[2] A similar median survival of 14.8 months has also been achieved with adjuvant photodynamic therapy (28% survival > 2 years; 22% survival > 5 years).[3–5]

The invasive growth pattern of GBM tumours, resulting in a poorly demarcated interface between tumour and normal brain tissues, represents a significant obstacle to effective treatment.[6] Incomplete surgical removal of all malignant cells is inevitable and recurrent tumours develop within 1–2 centimetres of the resection cavity in most patients.[6,7] Recurrent tumours are usually highly refractory to further radiation therapy and chemotherapy.[7,8]

Glioma invasion is a multi-step process in which tumour cells detach from primary lesions, establish new contacts with extracellular matrix (ECM) and neighbouring cells, degrade and/or remodel ECM barriers, and disperse into normal brain tissues. Glioma cells use myelinated nerve fibre tracts, vessel basement membranes and the subependymal layers as major routes of tissue invasion.[6] Several recent reviews have highlighted the important roles of ECM, proteinases, integrins and angiogenesis in glioma invasion and identified potential molecular targets for therapeutic intervention.[7,9–16]

This review focuses on the emerging role of "invadopodia" in invasion of glioma and other highly malignant

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tumours. The discovery of invadopodia as discrete ECM-degrading structures on the surface of several types of invasive and metastatic tumour cells has provided an important new insight into the cellular and molecular basis of tumour invasion.[17–25] Recent experiments showing that invadopodia can be disrupted at a molecular level to suppress the invasiveness of tumour cells *in vitro*, and more importantly, *in vivo*, has raised prospects that invadopodia could be targeted to produce novel anti-invasive therapies.[21,26]

2. Definition, discovery and markers of invadopodia

Invadopodia are actin-based protrusions of tumour cells and transformed cells which mediate proteolysis of ECM constituents, including fibronectin, laminins and collagens.[27,28] An *in vitro* matrix degradation assay (Fig. 1) is used to detect invadopodia activity.[25,29,30] Cells are grown on coverslips coated with fluorescently-labeled native ECM substrates (e.g. fibronectin) or with substitute proteins such as gelatin (denatured collagen). Digestion of matrices by invadopodia at discrete sites under the cells results in fluorescence-negative cavities which are detected by fluorescence confocal microscopy. Co-staining of cells for markers (e.g. cortactin, F-actin, phosphotyrosine) can provide further information about the temporal stages of invadopodia genesis and functional activity.[25,31]

Invadopodia were discovered in fibroblasts transformed by the *v-src* oncogene, which encodes a constitutively active non-receptor tyrosine kinase (vSrc). Src-transformed cells grown on fibronectin formed prominent ventral protrusions with adhesive[32] and degradative properties.[29] Additionally, vSrc was found to co-localize with the sites of fibronectin degradation.[33]

Invadopodia are distinct from other cell adherence sites such as focal contacts and focal adhesions which lack associated proteolytic activity and actin polymerization.[27]

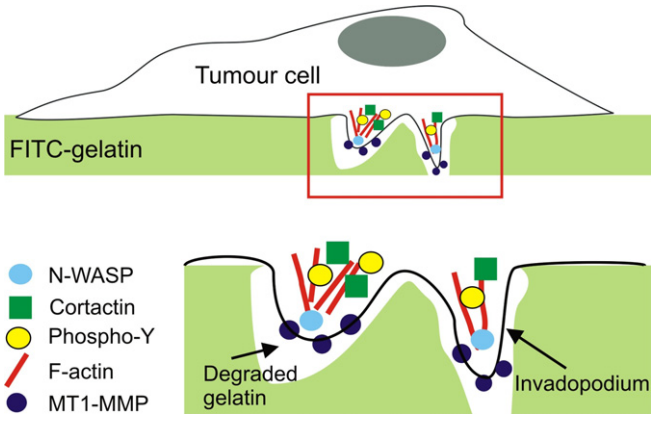

Fig. 1. Schematic representation of a tumour cell with ventral invadopodia degrading a film of fluorescein isothiocyanate (FITC)-labeled gelatin. Degradation of gelatin by invadopodia-associated gelatinases results in the formation of fluorescence negative cavities beneath cells that can be visualized by confocal fluorescence microscopy. Selected markers of invadopodia are shown. N-WASP, neural-Wiskott Aldrich Syndrome protein; MT1-MMP, membrane type-1 matrix metalloproteinase.

Invadopodia vary from 0.1 μm to 0.8 μm in diameter and may reach 2 μm or greater in length.[34] Electron microscopy showed that invadopodia can assemble into clusters around membrane invaginations proximal to the Golgi complex, suggesting that Golgi secretory functions could be important in invadopodia action.[35]

There is no single marker of invadopodia. However, invadopodia are often highly enriched with actin filaments (F-actin) and components needed for actin assembly including the Arp2/3 actin nucleation complex, neural-Wiskott Aldrich Syndrome protein (N-WASP) and cortactin.[17,18,25,36–38] Invadopodia are also major sites of tyrosine phosphorylation.[39] Indeed, co-localization of phosphotyrosine and cortactin is a highly predictive marker of active invadopodia that are degrading the matrix.[31] Notably however, invadopodia are transient structures with a variable composition. Indeed, elegant live cell kinetic studies have identified four distinct phases of invadopodia maturation based on: (i) localization of cortactin to ventral puncta, (ii) the recruitment of membrane type-1 matrix metalloproteinase (MT1-MMP) to these sites, (iii) the appearance of degraded foci in the underlying matrix, and (iv) the dissociation of cortactin from these sites.[25]

In addition to Src transformed fibroblasts, invadopodia have been definitively identified in cell lines or primary tumour cells from malignant melanoma (e.g. LOX, RPMI7951, A375MM)[22,24,35,40], breast/mammary carcinoma (e.g. MDA-MB-231, MTLn3)[23,25,36], glioma (e.g. SNB19, U251MG)[41,42] and head and neck squamous cell carcinoma (HNSSC)[37] and doubtless will also be found in other malignant tumour cells. We have also found evidence of robust invadopodia activity in a subset of primary GBM tumours (Fig. 2). Consistent with a direct role for invadopodia in cancer invasion, analysis of multiple breast cancer cell lines revealed a strong correlation between the invasive potential of cells *in vitro* and their ability to form invadopodia and digest a fluorescently labeled gelatin matrix.[19]

3. Invadopodia are closely related to podosomes

Several types of normal differentiated cells including osteoclasts, macrophages, dendritic cells, and endothelial cells, form actin-rich "podosomes" with close similarity to invadopodia. Podosomes exert adhesive and ECM degradation functions but have unknown protrusive activity whereas invadopodia are highly protrusive and can degrade the ECM although their adhesive activity has not been demonstrated[17,18,43] (see below). Despite similarities in molecular composition there may also be differences in organization, size, number and half-life of invadopodia and podosomes.[18,43]

Podosomes have a defined outer "ring" and central "core" organization with F-actin and actin regulators restricted to the core and integrins and associated proteins (e.g. paxillin, vinculin) present in the ring region.[17,18,34] It is not clear if this level of organization also exists in

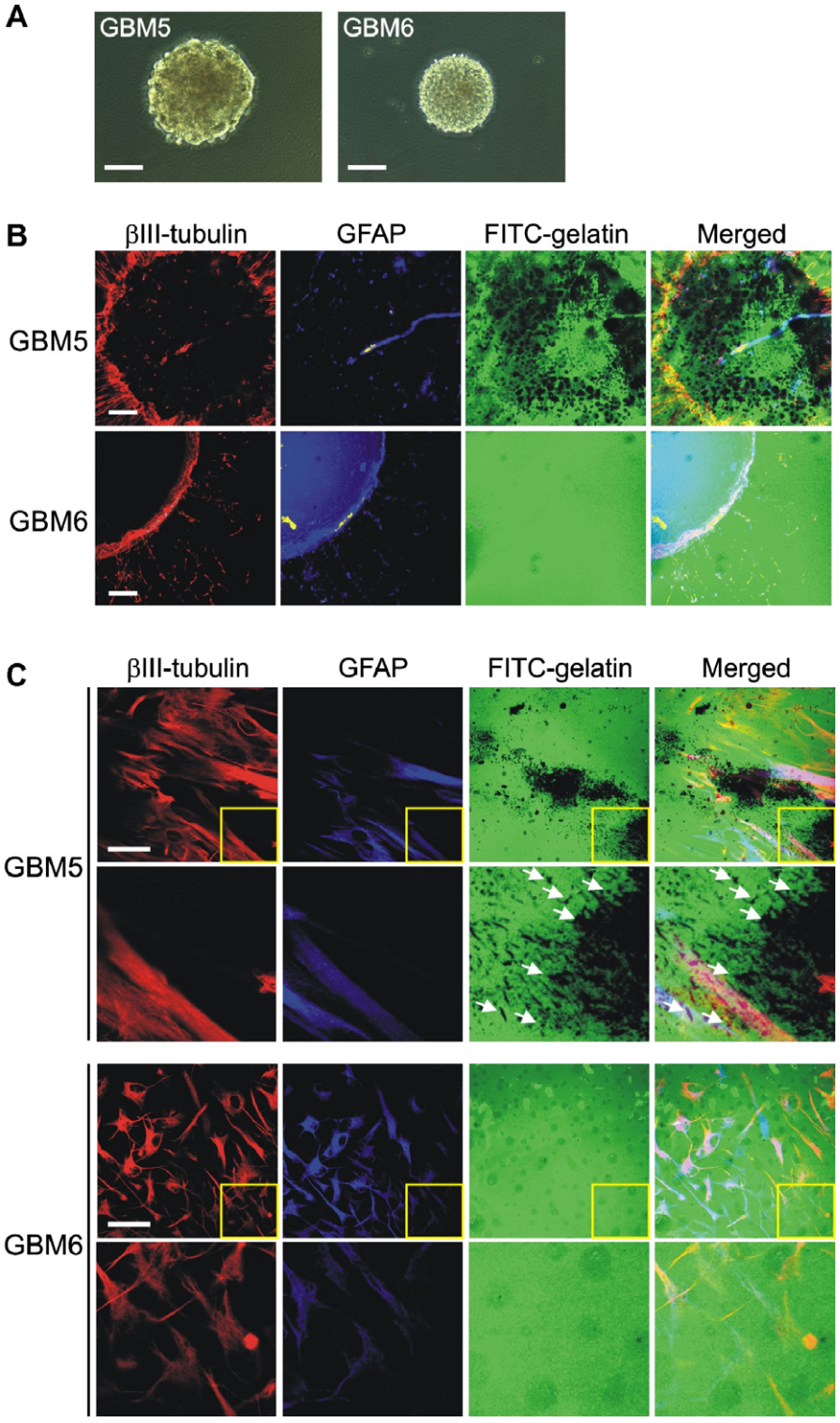

Fig. 2. Evidence of invadopodia in primary glioma cells. (A) Phase contrast images of glioblastoma multiforme GBM5 and GBM6 tumour spheres obtained by culturing World Health Organization grade IV gliomas from two patients as described.[127] Scale bar = 100 μm (B). GBM5 and GBM6 tumour spheres were seeded onto coverslips coated with fluorescein isothiocyanate (FITC)-gelatin (green) and 10 μg/mL laminin. Cells were fixed with 3% paraformaldehyde, permeabilized with Triton X-100, stained for βIII-tubulin (red) and glial fibrillary acidic protein (GFAP) (blue) and visualized by confocal microscopy. Fluorescence-negative (black) areas correspond to regions of invadopodia-mediated gelatin degradation and are associated with GBM5, but not GBM6 cells. Scale bar = 200 μm. (C). High power confocal microscopy images of GBM5 and GBM6-derived cells. Scale bar = 50 μm. Boxes indicate areas shown at high magnification below each panel. Arrows point to sites of invadopodia-mediated degradation. Melbourne Health Human Research Ethics Committee approval was granted for this work (HREC 2006.146).

invadopodia. In addition, podosomes are typically more numerous per cell and more rapidly turned over than invadopodia; a half-life of about 1 hour has been observed for invadopodia whereas podosomes turn over in 2 minutes to 10 minutes.[18,21] Functionally, podosomes are thought to have weaker ECM-degrading activity than invadopodia but this could also reflect faster turnover and smaller size.[18]

In Src transformed fibroblasts, podosomes typically assemble into visually striking doughnut-shaped arrays referred to as "rosettes". This phenotype is in part mediated by N-WASP.[44] Extensive podosome arrays are also found in osteoclasts and may contribute to formation of the osteoclast "sealing zone", which is thought to be intimately associated with bone resorption.

Podosome-forming "normal" cells may facilitate the invasion of tumour cells under some circumstances.[30,45,46] Breast carcinomas contain abundant tumour-associated macrophages, which secrete epidermal growth factor (EGF). EGF stimulates chemotaxis and invasion of breast tumour cells *in vivo*[45] and can enhance invadopodia formation *in vitro*.[21] Conversely, breast carcinoma cells secrete colony stimulating factor-1 (CSF-1) which promotes macrophage migration and podosome formation. This dual paracrine loop suggests a potential interaction of macrophages and breast cancer cells within the tumour microenvironment, which could dramatically increase migration of cells and ECM breakdown and thereby facilitate tumour invasion.

4. Molecular regulation of invadopodia

Multiple membrane-bound, cytoplasmic and secreted proteins regulate invadopodia (Fig. 3) and have functional roles in diverse cellular processes including cell adhesion, signal transduction, actin regulation, ECM proteolysis and membrane dynamics.[17,18,25,43]

4.1. Integrins

The adhesive properties of invadopodia are attributed primarily to integrins, a large family of heterodimeric transmembrane receptors comprising an α-subunit and a β-subunit which are also intimately associated with focal adhesions.[47] Integrin extracellular domains bind selectively to different ECM components. Intracellularly, integrins associate with several cytoplasmic linker proteins (e.g. α-actinin, vinculin and talin) which in turn physically engage with the actin cytoskeleton.[48] Upon binding to ECM substrates, integrins initiate signal transduction cascades, which modulate actin cytoskeletal organization. Focal adhesion kinase (FAK) and Src-family tyrosine kinases are important mediators of integrin signaling in focal contacts and focal adhesions, and are likely to also have roles in integrin signaling in invadopodia.[48,49]

The β1 integrin has been found to localize within invadopodia in Src transformed fibroblasts, LOX melanoma cells and MDA-MB-231 breast carcinoma cells.[20,24,34,50] In LOX cells cultured on collagen, α3β1 integrin forms a complex with the serine proteinase, seprase, suggesting a mechanism for integrin-targeted matrix degradation.[40,51] The α5β1 integrin, however, is enriched peripherally in invadopodia and is thought to stabilize invadopodia protrusion.[40] Ectopic stimulation of α6β1 integrin with laminin peptides or with β1 or α6 integrin stimulatory antibodies increased invadopodia activity and invasiveness of melanoma cells *in vitro*.[50] Activation of α6β1 integrin signaling was associated with increased tyrosine phosphorylation of p190RhoGAP (a GTPase activating protein for RhoA) and also enhanced p190RhoGAP localization in invadopodia.[51] These studies suggest that β1 integrin engagement with ECM ligands could be an important primary stimulus of invadopodia formation.

The β1 integrin is a component of the receptors for laminin, collagen and fibronectin, which are associated with perivascular invasion of glioma.[13] Levels of α2β1, α3β1, α5β1 and α6β1 integrins are elevated in GBM tumours relative to normal brain[52] with expression of α3β1 integrin also high at sites of human glioma infiltration in a mouse intracranial tumour model.[53] Inhibitory anti-β1 integrin antibodies can suppress invasion, migration and adhesion of primary glioma cells *in vitro*[54] while an antisense β1 integrin construct reduced the invasiveness of rat C6 glioma cells *in vivo*.[55] Whether these roles of β1 integrins in glioma invasion are specifically linked with invadopodia is unknown but this seems plausible.

Another integrin found at the invasive edge of high-grade glioma, together with its ligand, vitronectin, is αVβ3 integrin.[12,55,56] This integrin heterodimer is also found in the podosomes of osteoclasts.[57] Clustering of αVβ3 integrin together with MT1-MMP also occurs at discrete cell surface locations on breast carcinoma cells.[58] These domains could potentially correspond to invadopodia, although this has not been confirmed with matrix degradation assays. Processing of immature soluble MMP-2 into its active state by integrin-associated MT1-MMP stimulates collagen proteolysis by tumour cells.[58] αV integrin itself is also processed by MT1-MMP and enhances the motility of breast cancer cells on vitronectin.[59] Recognizing a potential role for αV integrins in glioma, a phase I clinical trial of the drug cilengitide (an αVβ3 and αVβ5 integrin inhibitor) was recently conducted in patients with recurrent tumours with mixed results.[60]

4.2. Src tyrosine kinase signaling

A central role for the Src non-receptor tyrosine kinase in invadopodia regulation has been inferred based on the ability of constitutively active Src to stimulate invadopodia formation in fibroblasts (which otherwise lack invadopodia) and breast carcinoma cells.[25,27,29,33] Indeed this activity of Src led to the discovery of invadopodia. In MDA-MB-231 breast cancer cells, expression of wild type or activated Src produced 4-fold and 7-fold increases respectively in the

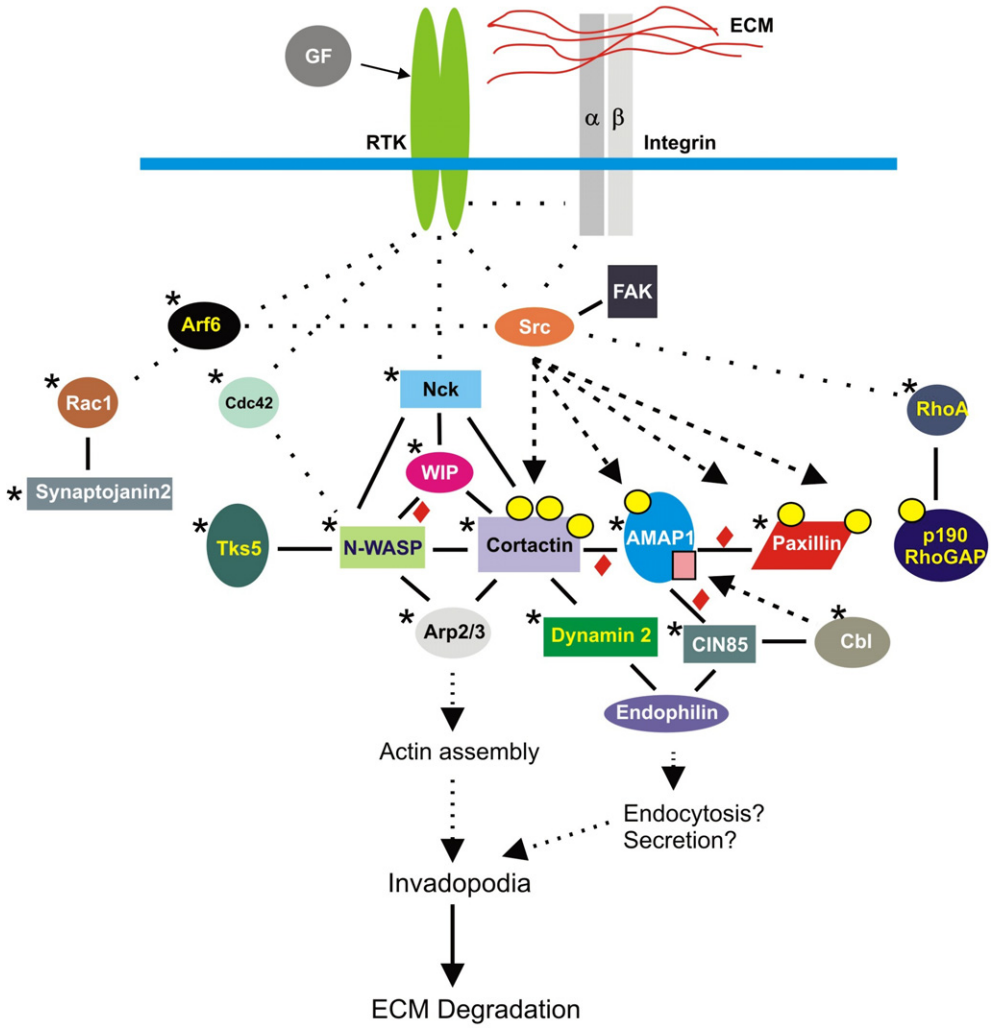

Fig. 3. Invadopodia signaling: a working model (see text for details). Integrins and receptor tyrosine kinases (RTKs) on invasive tumour cells are activated by adhesion to extracellular matrix (ECM) and growth factors (GF) respectively. Activation of Src (by integrins and/or RTKs) and recruitment and activation of multiple components of the actin regulatory machinery (cortactin, N-WASP, Arp2/3, Cdc42, Nck, WIP) promotes actin network assembly essential for invadopodia protrusion and matrix degradation. Potential roles for secretion and/or internalization are inferred based on the critical involvement of several membrane regulatory proteins in invadopodia regulation (e.g. Dynamin 2, endophilins, CIN85, Cbl and cortactin). Solid lines indicate direct protein-protein interactions and dotted lines indicate where the signaling intermediates are not known. Tyrosine phosphorylation (yellow circles) and monoubiquitination (pink squares) of substrates by Src and Cbl respectively, is indicated (dashed arrows). Proteins implicated as essential for invadopodia by RNA interference or dominant negative mutant studies are indicated (∗). Protein interactions that when disrupted in cells inhibit invadopodia formation are indicated (red diamonds).

mean number of invadopodia per cell and corresponding increases in matrix proteolysis.[25] The catalytic function of Src is required for this activity as kinase-inactive Src mutants fail to initiate invadopodia formation and matrix degradation or may function in a dominant negative manner to inhibit invadopodia.[25,31] Another Src-family member, Hck, is also capable of inducing podosome formation in fibroblasts.[61]

Several proteins linked to Src signaling are also enriched in invadopodia, not only in Src transformed cells, but also in many tumour-derived cell lines. Among them are the actin nucleation promoting factors, cortactin[20,25,31,62] and N-WASP,[21,36,38,63] the Arf GTPase activating protein (Arf GAP) AMAP1/ASAP1,[26,64] and the paxillin[20,65] and Tks5/Fish adaptor proteins.[24,66]

Manipulation of the expression levels or activities of these proteins by using RNA interference, dominant negative mutants or inhibitory antibodies, has confirmed that cortactin,[20,25,37] N-WASP,[21,38] AMAP1/ASAP1,[26,64] and Tks5/Fish[24] are required for invadopodia-mediated matrix degradation in a variety of tumour cell lines. Importantly, the inhibitory effects of depleting Tks5/Fish or AMAP1/ASAP1 on invadopodia formation were correlated with reduced invasive capacities of the cells in Matrigel basement membrane invasion assays.[24,26] Moreover, depletion of Tks5/Fish affected proteolysis-dependent but not proteoly-

sis–independent invasion in these assays showing that Tks5/Fish is not associated with "amoeboid-like" invasion.[24,67]

Further studies have demonstrated that cortactin overexpression is sufficient to stimulate increased invadopodia formation in HNSCC cells[37] and A375MM cells,[68] while in T47D breast cancer cells, which lack endogenous Tks5/Fish, actin-rich podosomes could be induced by co-expression of Tks5/Fish and activated Src, but not by activated Src alone.[24]

4.3. Cortactin

The involvement of cortactin in invadopodia formation is of particular interest. Cortactin is encoded by the *EMS1* oncogene on chromosome 11q13. *EMS1* gene amplification and/or cortactin overexpression occurs in a proportion of breast carcinomas and head and neck cancers and is correlated with tumour invasiveness and poor prognosis.[69] Consistent with this, cortactin has been shown to enhance the migratory and invasive capacity of fibrosarcoma and other cell types *in vitro*.[70–72]

Live cell imaging of Src-expressing MDA-MB-231 breast cancer cells showed that translocation of cortactin to "pre-invadopodia" is an early event in invadopodia initiation and precedes MT1-MMP recruitment to these sites and the subsequent degradation of underlying matrix substrates.[25] Studies in smooth muscle cells suggest that the cortactin SH3 domain mediates cortactin delivery to podosomes[62] and presumably this also occurs in invadopodia. The cortactin Src Homology 3 (SH3) domain binds to several proteins including N-WASP[69] and potentially this interaction could target cortactin to invadopodia. Both N-WASP and cortactin can activate the Arp2/3 complex to bind F-actin, creating nucleation sites for the synthesis of new actin filaments. Cortactin binds F-actin and Arp2/3 simultaneously and also inhibits actin debranching. This is thought to stabilise existing actin structures and drive new actin network assembly.[73] This activity of cortactin enhances lamellipodium "persistence" to promote directed migration[71] and may have a similar role in prolonging invadopodial protrusions.

It is important to note that cortactin-mediated stimulation of ECM degradation may not be mediated solely via effects on actin assembly and invadopodia. Overexpression of cortactin in HNSCC cells enhanced matrix proteolysis to a greater extent than could be accounted for simply by increased Arp3 and actin levels in invadopodia.[37,74] This effect of cortactin was however associated with an enhancement of MMP2 and MMP9 secretion and increased cell surface expression of MT1-MMP suggesting that cortactin also potently regulates secretory processes.[37,74]

4.4. AMAP1/ASAP1

It has been postulated that cortactin and other key invadopodial proteins form an "invasion complex" in tumour cells. Cortactin and paxillin have been found in association with AMAP1/ASAP1[26] and with protein kinase Cμ[20] in invadopodia. Surveys of breast carcinoma cell lines showed that these complexes were detectable in invasive but not non-invasive cell lines suggesting a link with malignancy.[20,26] Consistent with a direct role for a cortactin-AMAP1/ASAP1-paxillin trimeric complex in invasion, disruption of this complex in cells, either by expression of the paxillin-binding SH3 domain of AMAP1/ASAP1 or microinjection of peptides corresponding to the proline rich region that mediates binding of cortactin to AMAP1/ASAP1, inhibited invasion of MDA-MB-231 breast cancer cells through matrigel.[26] Even more remarkably, overexpression of the AMAP1/ASAP1 SH3 domain in mouse 4T1/luc breast cancer cells suppressed the formation of lung metastases in syngeneic Balb/c mice.[26]

AMAP1/ASAP1 has also been found in a complex with the CIN85 adaptor protein in invadopodia and moreover disruption of this interaction also perturbed invadopodia and inhibited breast cancer cell invasiveness *in vitro*.[75] CIN85 is a binding partner of Cbl E3 ubiquitin ligase, which mediates AMAP1/ASAP1 monoubiquitination. Monoubiquitination of the EGF and hepatocyte growth factor (HGF) receptors is an important signal for receptor downregulation via the endocytic pathway[76,77], but whether this modification regulates AMAP1/ASAP1 trafficking remains unknown. Surprisingly, the enzymatic function of AMAP1/ASAP1 appears to be dispensable for invadopodia formation as a mutant AMAP1/ASAP1 with a defective Arf GAP domain restored invadopodia formation in Src-transformed cells depleted of endogenous AMAP1/ASAP1.[64] This suggests that the main role of AMAP1/ASAP1 in invadopodia formation could be to serve as an adaptor protein.

4.5. Tks5/Fish

Tks5/Fish is a large adaptor protein with an N-terminal Phox homology (PX) domain, five SH3 domains and predicted Src phosphorylation sites.[78] The PX domain binds *in vitro* to the membrane lipids, phosphatidylinositol 3-phosphate [PI(3)P] and $PI(3,4)P_2$ and this interaction may facilitate Tks5 targeting to invadopodial membranes in Src transformed NIH3T3 cells.[66] The 5th SH3 domain of Tks5/Fish binds to members of the ADAM (A Disintegrin And Metalloproteinase) family[66] (discussed below) while the 3rd SH3 domain has been found to interact with another essential invadopodial component, N-WASP.[24] Precisely how these interactions impinge on invadopodia is not yet known.

4.6. Regulation of invadopodia by tyrosine phosphorylation

In general, phosphotyrosine levels in invadopodia correlate well with their matrix degradation activity.[31] However, it has become clear that several invadopodial proteins undergo tyrosine phosphorylation during invadopodia gene-

sis (e.g. cortactin, AMAP1/ASAP1, paxillin, p190RhoGAP) with differing consequences.

AMAP1/ASAP1 contains a predicted Src phosphorylation site at tyrosine 782 (Y782). A mutant form of AMAP1/ASAP1 lacking the Y782 phosphorylation site was unable to restore invadopodia in cells where endogenous AMAP1/ASAP1 was silenced with siRNA, whereas wild type AMAP1/ASAP1 was able to do so.[64] These results imply that Y782 phosphorylation is required for invadopodia formation. However, tyrosine phosphorylation of paxillin (at residues Y31 and Y118) appears to stimulate the turnover of mature invadopodia as a paxillin mutant lacking these two phosphorylation sites did not affect invadopodia formation but delayed invadopodia disassembly.[65]

Cortactin also contains tyrosine phosphorylation sites (at Y421, Y466 and Y482) and is highly phosphorylated on at least one of these residues (Y421) in podosomes and invadopodia.[79,80] Studies using purified recombinant proteins and *in vitro* actin assembly assays have provided an important clue to the role of cortactin tyrosine phosphorylation. Src-phosphorylated wild type cortactin more potently stimulated actin polymerization than a cortactin mutant lacking the three tyrosine phosphorylation sites.[81] This effect was maximal in the presence of N-WASP, WASP-interacting protein (WIP) and the Nck1 adaptor protein. All of these proteins have been independently linked to invadopodia formation and appear to form part of a dynamically regulated actin assembly complex.[21,25,38] Very recently it was confirmed that a cortactin mutant in which the Y421, Y466 and Y482 phosphorylation sites were mutated had a reduced capacity to induce invadopodia formation in A375MM melanoma cells compared to wild type cortactin.[68] Conversely, a cortactin mutant with the tyrosines substituted for glutamic acid (to mimic phosphorylation) enhanced invadopodia formation and matrix degradation compared to wild type cortactin. These findings show that Src phosphorylation of cortactin is a positive stimulus of actin network assembly and suggest this is tightly coupled to invadopodia activity.

4.7. Receptor tyrosine kinases

Two receptor tyrosine kinases, EGF receptor and the HGF receptor (c-Met), have been linked to invadopodia induction. EGF stimulation boosted invadopodia formation and ECM degradation in metastatic MTLn3 mammary carcinoma cells, whereas suppression of EGF receptor (EGFR) catalytic activity with the specific inhibitor AG1478 abolished this effect.[21] EGF induction of invadopodia was shown in RNA interference (RNAi) experiments to depend on the expression of endogenous N-WASP and Arp2/3 as well as several other components of the actin polymerization machinery including WIP, Nck1, and the Rho GTPase, Cdc42.[21] EGF-induced formation of invadopodia was also disrupted by overexpression of the N-WASP binding domain of WIP, but not with a distinct region of WIP that binds to cortactin[21] suggesting that interaction of endogenous WIP and N-WASP but not WIP and cortactin is critical for EGF signaling to invadopodia formation.

In LOX melanoma cells, HGF stimulation of endogenous c-Met receptors enhanced the capacity of cells to degrade matrix substrates, presumably via induction of invadopodia activity.[22] This activity of HGF was completely blocked by a dominant negative mutant of the small GTPase, ADP-ribosylation factor 6 (Arf6).[22]

It is not known if EGF and HGF receptors localize to invadopodia and thereby signal directly to the actin machinery and other target proteins located in invadopodia (e.g. Arf6) or whether these receptors activate invadopodia formation via intermediates such as Src. Pharmacological inhibitors or knockdown of Src expression could be used to examine this question. One interesting possibility is that EGFR and c-Met induce invadopodia via cross-talk with cell surface integrins.[82] The identification of EGF as a driver of invadopodia in breast cancer cells has important implications for glioma as EGFR is frequently overexpressed and mutated in high-grade tumours.[83] c-Met is also implicated in glioma invasion and its levels are upregulated in glioma together with its ligand HGF.

4.8. Rho-family GTPases

The Rho-family GTPases, Cdc42, Rac1 and RhoA, are key regulators of actin cytoskeletal organization and have essential roles in many cellular processes, including cell migration.[84,85] All three of these GTPases have also been shown to influence invadopodia regulation albeit in different cell types.[21,41,86] In SNB19 and U87MG glioma cells, depletion of Rac1 or its effector, synaptojanin 2 (a phosphoinositide phosphatase) using siRNA, inhibited invasiveness of cells through slices of rat brain tissue and through Matrigel.[41] At a cellular level, Rac1 and synaptojanin 2 silencing caused a marked decrease in lamellipodia and invadopodia formation. These effects appeared to be specific as endogenous synaptojanin 2 is enriched in both types of protrusions. These studies implicate a Rac1-synaptojanin 2 pathway in glioma cell lamellipodia and invadopodia formation that mediates invasion.

Silencing of Cdc42 expression also inhibited invadopodia formation in EGF-stimulated MTLn3 mammary carcinoma cells.[21] Although Cdc42 localization was not tested in this study, Cdc42 is reported to localize to podosomes in macrophages, where manipulation of its activity interfered with podosome organization.[87] Expression of a dominant active Cdc42 mutant was also capable of inducing matrix-degrading invadopodia in RPMI7951 melanoma cells.[88] These and previous studies showing that active (GTP-bound) Cdc42 can stimulate N-WASP and Arp2/3 dependent actin polymerization *in vitro*[89], support a role for Cdc42 in invadopodia actin network assembly.

In another study, endogenous active RhoA (RhoA-GTP) was shown to localize in protrusions of Src transformed NIH 3T3 fibroblasts. Co-incident staining of F-actin, cortactin and Tks5/Fish confirmed that these sites were podosomes.[86] Consistent with a functional role for RhoA in these cells, blockade of RhoA signaling using different strategies inhibited podosome formation and degradation of ECM substrates. Thus RhoA appears to have a role in invadopodia formation in Src transformed cells.

It is unlikely that Rac1, Cdc42 and RhoA would be simultaneously activated within invadopodia in any one cell type. RhoA and Rac1 have antagonistic activities and their activities are spatially and temporally regulated.[84] RhoA is locally inactivated in the lamellipodia of platelet derived growth factor-stimulated cells via a pathway that requires the RhoA negative regulator, p190RhoGAP.[90] Integrin signaling also promotes p190RhoGAP tyrosine phosphorylation and localization to invadopodia,[51] suggesting a mechanism by which RhoA could be locally downregulated in invadopodia. A clearer understanding of the dynamics of Rac1, Cdc42 and RhoA activation and downregulation in invadopodia will help to clarify the roles of these proteins.

4.9. Arf6

Arf6 is a Ras-superfamily GTPase involved in the regulation of plasma membrane recycling, exocytosis and actin polymerization.[91] Activation of Arf6 can induce the disassembly of adherens junctions in epithelial monolayers and initiate cell scattering (single cell migration), suggesting a possible role for Arf6 in epithelial mesenchymal transition (EMT) and tumour progression.[92,93] Arf6 is found in invadopodia in melanoma and breast cancer cells.[22,23] In LOX melanoma cells, HGF stimulation enhanced invadopodia activity which also coincided with activation of endogenous Arf6, whereas an Arf6 dominant negative mutant inhibited invadopodia formation.[22] Inhibition of the kinase MEK1 also efficiently blocked invadopodia suggesting that signaling via this kinase and the downstream ERK (extracellular regulated kinase) pathway is important for Arf6 activities.[22] Depletion of Arf6 with siRNA also blocked invadopodia formation in MDA-MB-231 breast cancer cells and in addition, inhibited the invasion of cells through Matrigel.[23] Moreover, Arf6 expression levels correlated with invasiveness in a panel of breast cancer cell lines and *in vitro* invasiveness of cells could be inhibited in all cases by Arf6 knockdown.[23]

Further studies are needed to define how Arf6 is regulated in invadopodia and to identify the effectors that mediate its biological activities. Potentially, Arf6 could be activated in invadopodia by Src signaling based on evidence that vSrc-induced scattering of MDCK cells can be blocked with an Arf6 dominant negative mutant.[92] Possible intermediates in such a pathway may include the Arf guanine nucleotide exchange factors (Arf GEFs), ARNO or EFA6.[93] Arf6 activation is coupled to the redistribution and/or activation of endogenous Rac1[93,94] but whether a similar pathway is triggered in invadopodia needs further investigation.

4.10. Dynamin 2

Dynamin 2 (Dyn2) is a large GTPase that regulates the scission of invaginating membranes to form endocytic vesicles while also regulating the actin cytoskeleton. Dyn2 localizes to invadopodia and podosomes in different cell types.[35,95] Consistent with a functional involvement of Dyn 2 in invadopodia regulation, expression of dominant negative Dyn2 mutants in A375MM melanoma cells resulted in a decrease in the number of ECM-degrading invadopodia,[35] while in another study, expression of Dyn2 mutants in Src transformed BHK21 cells either prevented podosome formation or delayed podosomal actin turnover.[95]

The mechanisms by which Dyn2 regulates invadopodia are not clear but several Dyn2 protein–protein interactions are important to consider. For example, Dyn2 can physically interact with cortactin[69] and depending on its concentration, can stimulate or inhibit cortactin-mediated actin assembly *in vitro*.[96] Dyn2-cortactin complexes have critical roles in the regulation of receptor-mediated endocytosis[97] and transport of proteins from the Golgi.[98] Thus, Dyn2-cortactin association in invadopodia could conceivably be coupled to secretory and/or endocytic pathways that require coordinated actin regulation.

Endophilin 2 also binds and co-localizes with Dyn2 in osteoclast podosomes[95] and with F-actin containing podosomes in Src transformed fibroblasts.[99] Endophilins are associated with the formation of clathrin-coated vesicles and an endophilin A2-dynamin endocytic complex has been specifically implicated in down regulation of MT1-MMP cell surface expression. In vSrc transformed cells, however, downregulation of MT1-MMP is impaired due to an increase in endophilin A2 phosphorylation at tyrosine Y315 and a resultant loss of affinity of endophilin A2 for dynamin.[99] This results in increased surface expression of MT1-MMP and higher matrix degradation capacity of cells.[99] Incidentally, endophilins also bind constitutively to another invadopodia-associated protein, CIN85.[75] Ligand-dependent ubiquitination and endocytosis of the EGF and c-Met receptors is mediated by an endophilin-CIN85-Cbl complex.[76,77] Potentially this complex could also regulate invadopodia through the ubiquitination and internalization of as yet unknown proteins.

4.11. Proteinases associated with invadopodia

Several metalloproteinases and serine proteinases are found in association with invadopodia and are likely to contribute to the ECM proteolytic activities of invasive tumour cells. The matrix metalloproteinases (MMPs) are a large family of Zinc (Zn)-binding endopeptidases that are classified on the basis of substrate specificity or subcellular

distribution as collagenases, gelatinases, stromelysins, metalloelastases or membrane-type MMPs (MT-MMPs).[100] Common structural features of MMPs include a signal peptide, a catalytic domain with a conserved Zn binding site and a hemopexin-like (PEX) domain.

The membrane tethered collagenase MT1-MMP and the secreted gelatinases, MMP-2 and MMP-9, localize to, or become activated at, invadopodia.[25,58,74,101–104] As discussed already, accumulation of MT1-MMP is likely to activate its substrate, MMP2, at these sites and stimulate focal matrix degradation.[25,58] The relevance of MT1-MMP in invadopodia has been tested with small interfering RNA (siRNA) and proteinase inhibitor experiments. In Src-expressing MDA-MB-231 cells, depletion of endogenous MT1-MMP or treatment with tissue inhibitor of metalloproteinase-2 (TIMP-2) had relatively little effect on pre-invadopodia formation (as assessed by cortactin recruitment to ventral membrane sites) but profoundly inhibited matrix proteolysis.[25] In HNSCC cells, MMP inhibitors inhibited invadopodia formation significantly suggesting that in these cells, matrix degradation products could provide a positive feedback signal that increases invadopodia activity.[37]

MMP-2, MMP-9 and MT1-MMP are highly expressed by human gliomas and their levels and enzymatic activity correlate with invasive behavior and/or grade of malignancy.[9,13,105–112] Consistent with a functional requirement for MMP-9 in glioma invasion, suppression of MMP-9 expression with antisense oligonucleotides impaired invasion of glioma cells *in vitro* and suppressed tumourigenesis *in vivo*.[113]

MMPs are involved not only in degrading ECM substrates but also in the enzymatic processing of a range of target proteins. CD44, a major receptor for the brain ECM component, hyaluronan, is a substrate of MT1-MMP. CD44 associates with the PEX domain of MT1-MMP in the lamellipodia of migrating cells. The CD44 cytoplasmic domain interacts with the actin cytoskeleton to target MT1-MMP to these sites.[114] The proteolytic products of CD44 released by MT1-MMP processing are known to stimulate tumour cell migration.[114,115] Very recently, CD44 has been found to be highly enriched in the podosomes of osteoclasts, identifying CD44 as the first non-integrin adhesion molecule in these structures.[116] Whether hyaluronan, the ligand of CD44, is also a target for invadopodia-mediated degradation is an interesting open question.

The ADAM metalloproteinases contain metalloproteinase, disintegrin, cysteine-rich and EGF-like domains followed by a transmembrane region and a cytoplasmic tail.[117,118] Several ADAMs interact with and modulate the function of integrins[117] and could therefore in principle regulate invadopodia functions such as ECM adhesion via this activity. ADAMs also function as "sheddases" to release and activate a range of transmembrane growth factors. ADAM19, ADAM12 and ADAM15 have been identified as Tks5/Fish-interacting proteins with ADAM12 also co-localizing with Tks5/Fish in podosomes.[66] These findings raise the interesting question of whether the Tks/Fish associated ADAMs contribute to the ECM degradation activity of invadopodia/podosomes or perhaps are involved in the specific processing of other target proteins (e.g. integrins).

The serine proteinases, seprase and dipeptidyl dipeptidase IV (DPP4) are closely related integral membrane proteins which co-localize to invadopodia in tumour cells.[40,103,119] DPP4 and seprase cleave matrix components such as collagen in addition to chemokines and other soluble factors.[119] These enzymes might confer the invasive properties of endothelial cells involved in capillary formation in invasive breast carcinoma.[120] DPP4 and seprase localized at podosomal protrusions of vascular endothelial cells and were also associated with regions of gelatin degradation. Additionally, a monoclonal antibody against the gelatin-binding domain of DPP4 prevented gelatin degradation by cells, thereby supporting a direct role for this enzyme in podosomes and potentially in tumour cell invadopodia.[120]

5. Opportunities for therapeutic intervention

In this review we have developed a working model of several key signaling pathways involved in invadopodia formation in tumour cells. An important question now is: can invadopodia-associated proteins be targeted to inhibit invasion and metastasis of tumours?

Independently of their role in invadopodia, MMPs were identified as promising targets for inhibiting tumour progression based on a strong correlation between metastatic potential and the capacity of tumour cells to degrade basement membrane ECM.[121] Several orally bioavailable "second generation" MMP inhibitors (MMPIs) have been evaluated in phase III clinical trials in patients with late stage cancers. However, none of the drugs proved beneficial and in some cases, they hastened disease progression. The failure of these trials may relate to the broad actions of the drugs resulting in inhibition of "anti-target" proteins with beneficial roles in host protection or homeostasis.[122] A more critical validation of MMPs as true anti-cancer targets is essential before new drugs can be evaluated.[122] Interestingly, MMP2 (which is predicted to be activated by MT1-MMP at invadopodia) has emerged as one of the best validated MMP anti-cancer targets in several aggressive cancers.[122] The challenge will be to develop third generation MMPIs with enough selectivity that will not interfere with host defence.[123]

Src family kinases, particularly Src itself, show increased activity in a range of human tumours (especially breast and colorectal cancers) and are viewed as attractive targets for anti-cancer therapies.[124] Several small molecule inhibitors of Src family kinases which compete for the ATP binding site and inhibit kinase activity have been developed and are undergoing preclinical testing or have entered phase I clinical trials for leukemias (e.g. CML)

and some advanced solid tumours (e.g. pancreatic carcinoma).[125,126] These inhibitors would be expected to potently inhibit invadopodia formation and invasiveness of tumour cells. However, Src kinases function as intermediates in diverse pathways and regulate many other processes including proliferation and survival in addition to cell migration and invasion. Consistent with this, Src kinase inhibitors suppress multiple aspects of tumourigenesis in xenograft tumour models including growth, metastasis, angiogenesis and survival.

Invadopodia are particularly sensitive to disruption *in vitro*; numerous studies show that a reduction in the expression levels of any one of several invadopodial components can abolish invadopodia formation.[20–26,37,75] This implies that cross-talk within invadopodial signaling networks is vital for invadopodia to function (Fig. 3). Strategies to deliver short interfering RNAs *in vivo* are being investigated for many diseases and potentially this approach will also provide a means to specifically disrupt invadopodia in the future.

In the meantime, interference with individual protein–protein interactions could be investigated as a way to target invadopodia, particularly as there is some evidence that certain invadopodial complexes are restricted to more highly malignant cells and may therefore represent unique cancer-related targets.[20,26]

The most compelling evidence that blocking protein–protein interactions in invadopodia could be used to inhibit tumour progression comes from studies showing that abrogation of AMAP1/ASAP1-mediated interactions mediated via its SH3 domain in 4T1 breast cancer cells suppressed the metastatic spread of these tumour cells in mice.[26] These experiments required transfection of a cDNA encoding the SH3 domain of AMAP1/ASAP1 into breast tumour cells and while this approach is not viable in a clinical setting, it may be possible to develop cell permeable small molecules (e.g. peptidomimetics or non peptides) which inhibit AMAP1/ASAP1 SH3 domain interactions, which could potentially be taken up by tumours. Agents that could interfere with other AMAP1/ASAP1 complexes, including those with cortactin[26] and CIN85[75] (which are mediated by proline-rich motifs in AMAP1/ASAP1) or that would disrupt N-WASP-WIP[21] complex formation, could also be investigated as targets for inhibiting invasion based on the evidence that these interactions are necessary for invadopodia formation or Matrigel invasion *in vitro*. Indeed, as details of the signaling pathways that control invadopodia continues to rapidly unfold, the scope for identifying novel anti-invasive targets in invadopodia should only increase in the future.

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