Chapter 21
Invadopodia: Interface for Invasion

Susette C. Mueller, Vira V. Artym, and Thomas Kelly

Abstract The term invadopodia is essentially a functional definition linking membrane protrusions with proteolysis of the matrix at focused points beneath cells cultured on two-dimensional ECM matrices. Their molecular definition awaits more complete characterization of the interactions of molecular components that organize into morphologically distinct structures recognized by characteristic actin/cortactin cores which subsequently attain a proteolytic functionality that facilitates cellular invasion. Because of their similarity to podosomes and because there are progressive stages in the formation of invadopodia, confusion exists regarding the precise definition of invadopodia. In this chapter, we discuss sources of confusion prevalent in the literature to date, delineate characteristic features of invadopodia, review molecular components required for invadopodia-mediated matrix degradation, and discuss the assembly of functional invadopodia and the relationship of the invadopodia membrane to the actin/cortactin core. Finally, we discuss what is known about the functional consequences of invadopodia-mediated matrix degradation and the role of proteases associated with invadopodia in cellular interaction with other cells and the matrix, and their contribution to the tumor microenvironment.

Introduction

Early studies of adhesion, migration, and invasion in Rous sarcoma-transformed cells revealed that the v-Src tyrosine kinase is transforming and produces a highly invasive cellular phenotype (Martin 2004, Thomas and Brugge 1997). In particular, the transformed cellular adhesions in fibroblasts that resulted from v-Src expression were termed invadopodia, rosettes, or podosomes and they displayed distinctive actin-associated morphology. Tumor cells were subsequently found to produce

S.C. Mueller
Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical School, Washington, District of Columbia 20057-1469, USA,
e-mail: mueller@georgetown.edu

D. Edwards et al. (eds.), *The Cancer Degradome*.

invadopodia, and they possess at least some of the characteristics described for transformed fibroblasts (Ayala et al. 2006, Chen 1989, 1990; David-Pfeuty and Singer 1980, Gavazzi et al. 1989, Kelly et al. 1994, Wang et al. 1984, Weaver, 2006, Yamaguchi et al. 2006). Invadopodia were originally named and described based on the ability of invadopodial membranes to penetrate the extracellular matrix (ECM) and their association with sites of localized degradation in fluorescent matrices (Chen 1989). In summary, invadopodia adhesion sites in tumor cells are recognized by dot-like aggregates of actin and cortactin, and their membranes penetrate the matrix in the form of filopodia-like extensions assisted by membrane-associated proteolytic enzymes. Localized matrix-degradation, podosomes, and invadopodia have been reviewed recently (Ayala et al. 2006, Calle et al. 2004, Chen et al. 1994, Chen 1996, Linder 2007, McNiven et al. 2004, Weaver 2006, Yamaguchi et al. 2006, Yamaguchi and Condeelis 2007).

Invadopodia or podosomes have been reported in fibroblasts, epithelial and mesenchymal tumor cells, and also in endothelial cells, smooth muscle cells, and hematopoietic cells, the latter primarily of the monocyte lineage. Podosomes and invadopodia all appear to be Src dependent (Martin 2004). However, confusion exists as to the distinction between podosomes and invadopodia (for review, *see* Ayala et al. 2006, for example). There is no single antibody that uniquely and exclusively identifies invadopodia or podosomes. A list of proteins, compiled based on a variety of data, details possible constituents of podosomes, invadopodia, and circular dorsal ruffles (Buccione et al. 2004), but the criteria for the identification of invadopodia and the types of experiments used to support the conclusions concerning them have not been uniformly agreed upon from study to study.

Confusion between these structures arises from a variety of other factors as well. While they have overlapping constituents and morphological features, there appear to be distinct differences from cell to cell with regard to number and size, association of internal and external membranes with the actin core, and molecular constituents such as integrin heterodimers (compare epithelial cell podosomes from normal cells to macrophages to tumor cell invadopodia, e.g., Artym et al. 2006, Clark et al. 2007, Evans et al. 2003, Spinardi et al. 2004 and in reviews mentioned above). Another major difference relates to cellular response to ECM. Adhesions, including invadopodia, do not attain full functionality unless the cell receives proper signals from the ECM (e.g., Cukierman et al. 2001, Mueller et al. 1999), and deregulation of integrins can promote invasiveness via invadopodia (Nakahara et al. 1996, 1998). For example, the ECM-degrading enzyme fibroblast activation protein-α (FAP or seprase) directly interacts with $\alpha 3\beta 1$ integrin at invadopodia, but only when cells are cultured on collagen and not plastic (Mueller et al. 1999). Thus, one cannot assume that podosomes or invadopodia in cells cultured on plastic are the same morphologically or functionally as those observed when cells are cultured on 2D or 3D ECM proteins.

Other sources of confusion arise from the use of different cell types with different signal transduction backgrounds combined with inadequate criteria for invadopodia identification. Actin core assembly might proceed, and proteolytic function coupling might lag behind or not be present when cells are stimulated

with a given growth factor or pharmaceutical agent. For example, phorbol ester treatment of A7r5 smooth muscle cells elicits podosomes (actin core formation) but not matrix degradation; in contrast, the same phorbol ester elicited both actin core formation and matrix degradation in primary vascular smooth muscle cells (VSMC) (Furmaniak-Kazmierczak et al. 2007). Although the primary role of podosomes is thought to be the mediation of adhesion and migration in hematopoietic cells, their role in localized matrix degradation, similar to invadopodia, has recently been established using tests for localized matrix degradation in vitro (Table 4.1). And, an invasive function for podosomes in leukocytes has recently been proposed (Carman et al. 2007).

We will focus in this chapter on the role of invadopodia in localized ECM degradation emphasizing what is known about the stages of their formation and their role during tumor invasion and metastasis.

Molecular Components of Invadopodia

Molecular components of invadopodia that have been studied in conjunction with localization of MT1-MMP protease, or with sites of localized degradation in fluorescent matrices, are listed in Table 4.1.

Invadopodia components, in general, fall into four classes of molecules: (1) proteins involved with actin polymerization, (2) proteins involved with integrin-mediated adhesion, (3) signaling proteins regulating actin polymerization and membrane remodeling (kinases and Ras-related GTPases), and (4) ECM-degrading proteases (Artym et al. 2006, Ayala et al. 2006, Buccione et al. 2004, Gimona and Buccione 2006, Linder 2007, Weaver 2006 Yamaguchi and Condeelis 2007). Many of these components play important roles in lamellipodia formation, cell spreading, and dorsal ruffling in response to growth factor, and some are found in focal adhesions. Many more components have been implicated in podosome formation and stability including microtubule-related HDAC activity and KIF1C proteins, for example, but their involvement in protease delivery or invadopodia-mediated matrix degradation are not known (Linder 2007). Rather than reviewing all the potential components of invadopodia, since these are covered well in the numerous reviews mentioned above, we will focus on reviewing papers in which the protease activity of invadopodia has been considered including the impact of signaling molecules on the functional outcome of invadopodia formation.

Identification of Invadopodia

To provide a framework for understanding the cellular signaling required for the formation of functional invadopodia, it is necessary to discuss the steps in their genesis and to further define criteria for their identification.

Membranes of Invadopodia

Invadopodia are proteolytically active membrane protrusions that adhere to and interact with the ECM. The membrane protrusions closely resemble filopodia in size and they are closely associated with actin/cortactin cores (Fig. 21.1). Electron microscopy of invadopodia from MDA-MB-231 breast cancer, LOX and A375 melanoma, and Rous sarcoma virus transformed chicken embryonic fibroblasts cells invading into thick matrices indicates that invadopodia are of approximately the same dimension as filopodia, as thin as about one to several hundred nanometers in diameter but many microns in length, thus distinguishing them from other protrusions, such as larger cellular protrusions, pseudopodia, or lamellipodia (Fig. 21.1; Bowden et al. 1999, 2006; Chen 1989, Coopman et al. 1996, Gimona and Buccione 2006, Kelly et al. 1994, Monsky et al. 1994, Mueller et al. 1992, Mueller and Chen 1991). Other immunofluorescence studies have illustrated invadopodial extensions of slightly wider dimensions, on the order of microns (e.g., Bourguignon et al. 1998, Hauck et al. 2002, Hiratsuka et al. 2006). An interesting feature highlighted in studies using the N-WASP biosensor to detect localization of N-WASP activity at sites of degradation in MTLn3 cells cultured on fluorescent matrix was that the penetration was progressive, with the hole in the matrix broadening and lengthening over time [*see* Fig. 21.1 (Lorenz et al. 2004)]. N-WASP activity is high in early stages of matrix degradation and the holes start out at something less than 2 μm in diameter, but progressively N-WASP activity decreases as the hole is deepened and widened.

Correlative light and electron microscopy of A375MM melanoma cells was used by Baldassarre et al. (2003) to describe an "extracellular matrix-degrading structure" (EDS) that included invaginations of up to 8 μm width associated with gelatin fragments (*see* Fig. 21.1). Surface protrusions associated with them were several hundred nanometers to several micrometers in diameter. In breast cancer cells, invadopodial actin/cortactin cores were ~0.5–1 μm in diameter, similar in size to the holes in the matrix formed under them on 2D gelatin films which start out less than 1 μm in diameter and become wider (Artym et al. 2006, unpublished data). These were detected on films that were just under 50 nm thick in contrast to the much thicker matrices evidenced in vertical confocal sections in the N-WASP study discussed above (Lorenz et al. 2004). In the latter study, the use of time lapse and confocal sectioning was sufficient to differentiate early steps from later steps in cellular invasion. However, Z-depth resolution in detecting invadopodia-mediated matrix degradation becomes an issue, as the sensitivity of detection is decreased with increasing thickness of the matrix, and is inherently of a lower resolution than in the *X*–*Y* axis. Thus, in the absence of time lapse imaging, and on matrices of variable thickness, early steps in formation of invadopodia could be potentially confused with later steps.

As cells invade into a thick layer of matrix, what becomes of the initial invadopodium relative to the leading edge of the cell moving into the matrix? Martins et al. have reported that the formation of filopodia occurs over the entire

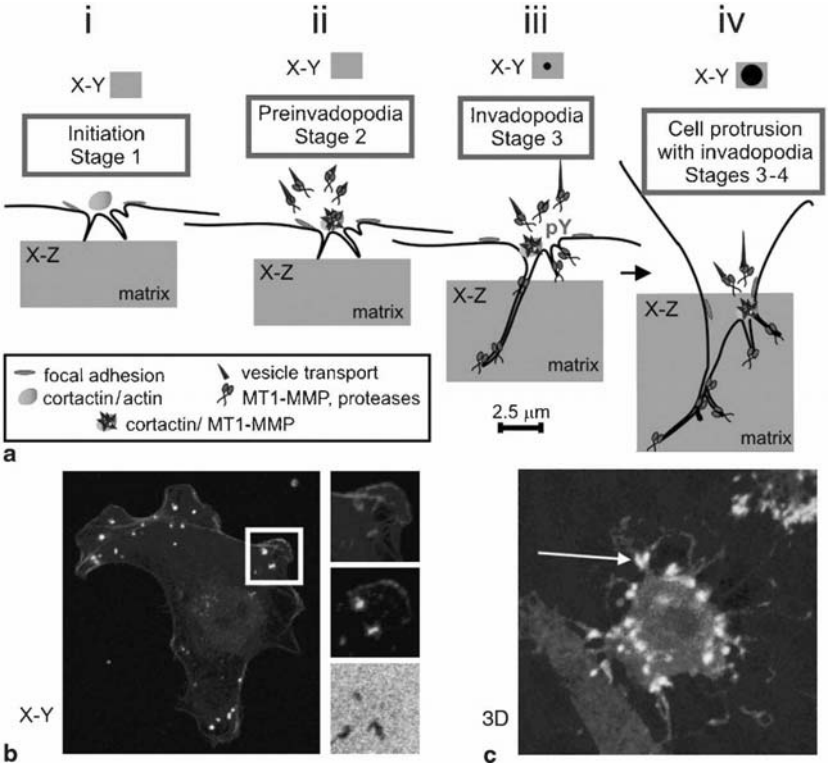

Fig. 21.1 Stages of invadopodia in 2D and 3D culture settings. **a** *i–iv* illustrate the initial formation of invadopodia beginning with cortactin recruitment (**i**), followed by recruitment of proteases such as MT1-MMP (**ii**), extension of invadopodia membrane into matrix facilitated by membrane-associated proteases and (**iii**), the enlargement of the site of degradation and invasion as the cell extends a pseudopodia/protrusion containing invadopodia and adhesion sites. **b** MDA-MB-231/c-Src(Y527F) breast cancer cells were immunostained using anticortactin 4F11 mAb (*green*) and counterstained with phalloidin (*red*) and imaged using confocal microscopy. The gelatin matrix (*grey*) contained sites of degradation and colocalizing F-actin and cortactin (*contained in insets*). **c** MDA-MB-231/c-Src(Y527F) cells were cultured on a thicker gelatin matrix whose autofluorescence is visualized together with cortactin in the red channel, DAPI in the blue channel, and antiphosphotyrosine 4G10 mAb staining in the green channel of this confocal image. Invadopodia are seen projecting into the matrix around the cell and aggregates containing colocalized cortactin and phosphotyrosine are visualized as yellow dots (*arrow*) (*See also* Color Insert I)

cell surface when endothelial cells are first introduced into a three-dimensional (3D) collagen gel culture (Martins and Kolega 2006). However, the focus of their formation increasingly localizes to the so-called peripheral zones of pseudopodial branches. Intermediate pseudopodia, the source of the pseudopodial branches, were described to be 2–5 μm in width. But, the relationship of the filopodia that formed in response to the matrix in this study and invadopodia is not clear as no markers for invadopodia such as cortactin, phosphotyrosine (pY), Tks5/FISH, or MT1-MMP

were examined (*see* Table 21.1). On the contrary, this study highlights that there is a hierarchy of protrusion formation with fine filaments associated with the ends of branched pseudopodia. Thus, some of the confusion in the literature is at least partially due to questions of whether invadopodia (less than~0.5 μm) or pseudopodial branches (2–5 μm) were examined. There is a dearth of high-resolution studies utilizing a number of invadopodial and focal adhesion markers to identify pseudopodia, which might contain invadopodia together with focal or matrix adhesions. An interesting question with regard to the role of focal adhesion kinase (FAK) in invadopodia is its colocalization with Src and integrins in protrusions of v-Src transformed fibroblasts (Hauck et al. 2002): Do these structures contain cortactin and MT1-MMP aggregates in the same sites? This appears to be an unresolved question and conflicts with the situation in breast cancer cells where FAK localizes to focal adhesions but not phosphotyrosine-rich aggregates resembling invadopodia complexes (Bowden et al. 2006).

The complexity of the leading edge of the cell in contact with the ECM has also been illustrated by the ECM-degradation structure (EDS) or degrading structures described by Baldassarre et al. (2003) using electron microscopy. Their images of invading cells in 3D collagen gels reveal fine protrusions similar in size to invadopodia (Wolf et al. 2007 and references therein, Burgstaller and Gimona 2005, Furmaniak-Kazmierczak et al. 2007). The data can be interpreted collectively to suggest that invadopodia form in association with larger cellular protrusions and at the leading edge of the cell (Fig. 21.1a, *iv*). When cells are cultured on extremely thin matrixes, the insertion of the invadopodia into the matrix is thwarted by glass and perhaps, consequently, protrusion size remains limited.

On 2D substrates, invadopodia are often formed under the cell, and the resolution at the light microscope level, even using a confocal microscope, is not sufficient to visualize the localization of the membranes associated with actin/cortactin cores. Simultaneous imaging of invadopodial membranes together with the adhesive, actin-rich core is typically not reported, and is technically difficult as the membranes of invadopodia have no unique markers. However, a number of transmembrane or membrane-bound proteins have been localized to actin/cortactin cores associated in the same study with sites of localized degradation of the fluorescent matrix. These include ADAM 12 in Src-transformed NIH 3T3 cells (Abram et al. 2003), CD44 in Hela cells (Vikesaa et al. 2006), FAP/seprase (Mueller et al. 1999), uPAR (Furmaniak-Kazmierczak et al. 2007), α3β1 integrin (Mueller et al. 1999), β1 integrin (Mueller and Chen 1991), and MT1-MMP (Artym et al. 2006, Furmaniak-Kazmierczak et al. 2007). In 2D studies, these all appear dot-like; however, the ultrastructure of the membranes at these sites is not known. Thus, the membrane could be an invadopodia protrusion, or, alternatively, these membrane proteins might be concentrated within vesicles associated with the invadopodia. In v-Src transformed chicken embryo fibroblasts, immunoelectron microscopy reveals the association of β1 integrins with the membrane extensions and vacuoles, again highlighting the complexity of the invadopodial structure in cells invading into 3D matrices or thick 2D substrata (Mueller and Chen 1991).

Table 21.1 Stages of invadopodia formation and function and their molecular components

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
I–III	Actin	Dynamic actin is associated with invadopodia.	Baldassarre et al. 2006, Yamaguchi and Condeelis 2007
I–III	ADAM 12	Colocalized with invadopodia and sites of localized degradation in breast tumor cells and v-Src transformed fibroblasts; associated with Tsk5/FISH.	Abram et al. 2003, Courtneidge et al. 2005, Huovila et al. 2005, Seals et al. 2005
I–II	AFAP-110	See PKCα entry	Gatesman et al. 2004
None	AP-2	No evidence that AP-2 was involved with invadopodia in glioblastoma cells. siRNA targeting AP-2 blocked endocytosis but not phagocytosis.	Chuang et al. 2004
I–III	Arf6/Erk	Arf6 expression was correlated in breast cancer cell lines to invasion and gelatin degradation which could be blocked by siRNA targeting Arf6. Arf6-eGFP, but not Arf1-eGFP, localized at the degraded holes. HGF stimulated invadopodia formation in LOX and paxillin, Arf6, and holes colocalized.	D'Souza-Schorey and Chavrier 2006, Hashimoto et al. 2004, Hoover et al. 2005, Tague et al. 2004
I–III	Arp2/3	siRNA of components of Arp2/3 complex members blocked formation of invadopodia and localized matrix degradation. Components localized to invadopodia actin cores.	Yamaguchi et al. 2005
I–III	ASAP1/AMAP1	Required for cortactin/actin cores and invadopodia-mediated matrix degradation in breast cancer cells. Recruited cortactin and paxillin downstream of Arf6.	Hashimoto et al. 2006, Onodera et al. 2005
I–III	CD44/IMP1 and IMP3	In the Met-1 tumor line, CD44v3,8–10 associated with MMP-9 in invadopodial protrusions. In Hela cells, IMPs stabilized CD44 mRNA as shown by siRNA knockdown of IMP1 and 3. Knockdown blocked colocalization of actin cores and CD44 as well as matrix degradation.	Bourguignon et al. 1998, Vikesaa et al. 2006
I–III	Cdc42, RhoA	In breast cancer cells, Cdc42 is required for invadopodia and localized matrix degradation: demonstrated by siRNA knockdown. In melanoma cells, dominant negative (d.n.) Rho did	Furmaniak-Kazmierczak et al. 2007, Hai et al. 2002, Lener et al. 2006, Linder, 2007, Moreau et al. 2003, 2006, Nakahara et al.

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Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
		not inhibit invadopodia degradation activity, but d.n. Rac, Cdc42, or frabin, a Cdc42 effector, did inhibit. Of the latter group, activated forms increased degradation. Cdc42 promoted podosome formation in primary aortic endothelial cells (actin-dots).	2003, Tatin et al. 2006, Varon et al. 2006, Webb et al. 2005, Yamaguchi et al. 2005
I–III	Cin85	Cin85 was required together with Cbl for invadopodia-mediated matrix degradation downstream of AMAP1 (ASAP1). Cin85 colocalized at holes and with AMAP1 on gelatin films.	Nam et al. 2007
I–III	Cofilin	siRNA reduced the population of immobile, long-lived invadopodia (actin cores) and reduced the number of cells with localized degradation.	Yamaguchi et al. 2005
I–III	Cortactin	In osteoclasts, siRNA targeting did not inhibit cell differentiation on bone, but prevented formation of sealing rings and bone resorption; wild type and SH3 deleted, but not 3Y mutant cortactin reconstituted podosome formation after siRNA knockdown. In breast, head, and neck squamous carcinoma: siRNA eliminated actin cores and localized gelatin degradation; colocalization with MT1-MMP and holes was demonstrated. Cortactin was required for protease secretion and surface membrane levels of MT1-MMP. Original description of stages of invadopodia formation based on time course experiments was proposed. In A7r5 smooth muscle cells and NIH 3T3 cells, active Src and PMA induced podosomes that required cortactin; 3Y mutant was translocated to the cell surface and endogenous pY cortactin was still present at podosomes. Cortactin is required for invadopodia and matrix degradation in fibroblasts; 3Y mutant of cortactin failed to rescue cortactin siRNA depletion.	Artym et al. 2006, Clark et al. 2007, Tatin et al. 2006, Tehrani et al. 2006, Varon et al. 2006, Webb et al. 2007, Zhou et al. 2006

Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
I–III	Dynamin	In human melanoma cells, dynamin mutants (GTPase inactive or deltaPRD domain) blocked localized matrix degradation. Actin, cortactin, and pY colocalized with dynamin at holes.	Baldassarre et al. 2003, Buccione et al. 2004, McNiven et al. 2004
I–III	EGF	Increased invadopodia activity in breast cancer cells with elevated EGFR.	Yamaguchi et al. 2005
I–III	ERK	Erk localized to actin punctae; U0126 inhibited but Src(Y527F) induced matrix degradation in VSMC. In LOX melanoma cells, phospho-Erk localized to sites of degradation. See also Arf6/Erk.	Furmaniak-Kazmierczak et al. 2007, Tague et al. 2004
II–IV	FAP/seprase	FAP/seprase, a serine-type protease, interacted with integrins and associated with invadopodia in fibroblast, endothelial, and melanoma cells. In fibroblasts, DPPIV colocalized with FAP/seprase in invadopodia of cells embedded in 3D collagen. FAP/seprase has not been localized to actin/cortactin cores.	Artym et al. 2002, Chen and Kelly 2003, Ghersi et al. 2002, 2006, Goodman et al. 2003, Kelly, 2005, Monsky et al. 1994, Mueller et al. 1999
I–II	Glycerol-phospho-inositols	These compounds blocked localized matrix degradation in breast and melanoma cancer cells.	Buccione et al. 2005
None	Grb2	Grb2 siRNA had no effect on breast cancer cell invadopodia formation and GFP-Grb2 did not localize to invadopodia.	Yamaguchi et al. 2005
I–III	HGF	Stimulated invadopodia formation in LOX melanoma cells determined by localized degradation.	Tague et al. 2004
II–III	High pY	Colocalization of pY and cortactin or phosphocortactin in Src-activated cells identified invadopodia at sites of matrix degradation.	Bowden et al. 2006, Furmaniak-Kazmierczak et al. 2007
II–IV	MMP-2	Exogenous MMP-2 and anti-MMP-2 staining localized to sites of degradation in v-Src transformed fibroblasts. Anti-MMP-2 staining was not detected in invadopodia of VSMC, although uPAR, MMP-9, and MT1-MMP were.	Furmaniak-Kazmierczak et al. 2007, Galvez et al. 2002, Monsky et al. 1993
II–IV	MMP-9	Localization of anti-MMP-9 at Src (Y527F) induced invadopodia of VSMC.	Furmaniak-Kazmierczak et al. 2007

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Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
II–IV	MT1-MMP	Transfection of CHO or MCF-7 tumor cells with MT1-MMP was sufficient to induce invadopodia-mediated matrix degradation. MT1-MMP siRNA and GM6001 inhibited TGFα-induced invadopodia in primary aortic endothelial cells. MT1-MMP was required for invasion into collagen by a number of cell types. Also required for collagen degradation and phagocytosis by human gingival fibroblasts. High resolution confocal imaging colocalized MT1-MMP, F-actin, and sites of degradation in 2D and 3D cultures. Many other 3D studies have imaged integrin, MT1-MMP, and F-actin on the cell surface of invading cells. In 2D, it was present with cortactin prior to formation of sites of degradation and lingered after cortactin has disengaged in invadopodia in breast tumor cells; was required for localized degradation in melanoma cells; colocalized with pY-cortactin at sites of matrix degradation in VSMC.	Artym et al. 2006, d'Ortho et al. 1998, Furmaniak-Kazmierczak et al. 2007, Hotary et al. 2003, Itoh and Seiki 2006, Lee et al. 2006a, Nakahara et al. 1997, Sabeh et al. 2004, Strongin, 2006, Taniwaki et al. 2007, Varon et al. 2006, Wolf et al. 2007, Wolf and Friedl 2005, Yana et al. 2007
I–III	Nck1	GFP-Nck1 was localized at invadopodia of breast cancer cells. siRNA targeting Nck1 blocked actin core formation and matrix degradation and rescue with wild-type Nck1 partially restored invadopodia.	Yamaguchi et al. 2005
I–III	N-WASP	siRNA-mediated knockdown demonstrated that N-WASP was required for invadopodia formation and localized degradation in mammary carcinoma cells. Mutants of N-WASP that were unable to activate the N-WASP effector Arp2/3 complex blocked podosome formation in v-Src-transformed 3Y1 rat fibroblasts and fibronectin degradation (i.e., invadopodia).	Chen, 1989, Lorenz et al. 2004, Mizutani et al. 2002, Yamaguchi et al. 2005

Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
I–III	p190RhoGAP	Colocalized with actin at invadopodia and signaling through beta 1 integrin in melanoma cells resulted in p190RhoGAP tyrosine phosphorylation. Microinjected anti-p190RhoGAP antibodies inhibited localized matrix degradation.	Nakahara et al. 1998
None	P38 MAPk	The MAPk inhibitor SB203580 failed to block Src(Y527F)-induced invadopodia in VSMC.	Furmaniak-Kazmierczak et al. 2007
III–IV	p61Hck	In macrophages, p61 Hck, Src-family member tyrosine kinase, localized in lysosomes and induced podosomes when constitutively activated. In NIH3T3 cells, it induced rosettes of podosome with localized gelatin degrading activity.	Cougoule et al. 2005
I–III	PAK	Activated or open conformation of kinase dead PAK induced invadopodia and localized matrix degradation in VSMC. PAK localized to holes of degraded gelatin.	Furmaniak-Kazmierczak et al. 2007
I–III	Paxillin	Localized together with cortactin to sites of matrix degradation in breast cancer and melanoma cells.	Bowden et al. 1999, Tague et al. 2004
II–IV	PI3K	PI3K inhibitors blocked matrix degradation in melanoma cells and primary aortic endothelial cells. Wortmannin blocked hole formation and also blocked colocalization of MMP-9 with actin cores.	Nakahara et al. 2003, Redondo-Munoz et al. 2006, Varon et al. 2006
I–III	PKC	PMA-induced podosomes were similar to Src-induced podosomes in HUVEC cells and were associated with matrix degradation. Src-induced podosomes, that is, invadopodia, required MT1-MMP, PKC, Src, and Cdc42. Immunofluorescence colocalization and a time course demonstrated transient induction of podosomes after PMA treatment. The phorbol ester PDBu induced podosomes in VSMC and A7r5 cells, but not degradation in the latter.	Burgstaller and Gimona 2005, Furmaniak-Kazmierczak et al. 2007, Hai et al. 2002, Lener et al. 2006, Webb et al. 2005, Zhou et al. 2006

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Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
I–II	PKCα	Constitutively activated PKCα or PMA activated c-Src and podosome formation in an AFAP-110- dependent manner in ovarian carcinoma cells. In Src(Y527F)-activated fibroblasts, AFAP-110 was required for podosome formation. Degradation was not examined.	Gatesman et al. 2004
I–III	PKD1 (PKCμ)	A complex of PKD1, paxillin, and cortactin was formed in invasive breast cancer cell lines and its role in invadopodia was suggested by colocalization of its components with invadopodia at sites of degradation.	Bowden et al. 1999
II–IV	Rab8	In MDA-MB-231 breast cancer cells, MT1-MMP and Rab 8 colocalized at the cell surface. Effects of Rab8 mutants on collagen gel invasion and thick gelatin films was noted: Rab 8 inhibited and its dominant negative mutants stimulated delivery of MT1-MMP to the cell surface.	Bravo-Cordero et al. 2007
I–III	Rac	D.n. Rac1 blocked Src(Y527F)-induced invadopodia in VSMC. Rac1 is required for peripheral cortactin localization in fibroblasts. However, d.n. Rac1 had no influence on PMA-induced podosomes in HUVEC, whereas d. n. Cdc42 and d.n. RhoA blocked their formation (number of cells with podosomes).	Furmaniak-Kazmierczak et al. 2007, Tatin et al. 2006, Weed et al. 1998
I–III	Rac1/ synaptojanin	Number of glioblastoma cells degrading the matrix was reduced by siRNA targeting Rac1 or synaptojanin 2 (downstream of Rac1). Synaptojanin 2 localized to sites of degradation.	Chuang et al. 2004
I–III	Rho	In NIH 3T3 cells transfected with activated Src(Y527F), activated Rho colocalized with F-actin, cortactin, and Fish and it was necessary for podosome structure and localized degradation. However, d.n. Rho had no effect on localized degradation in melanoma cells.	Berdeaux et al. 2004, Nakahara et al. 2003

Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
I–III	Src	Src is required for invadopodia formation and degrading activity in breast cancer cells and fibroblasts. Constitutively, active Src(Y527F) promoted podosomes and invadopodia formation in multiple cell types.	Artym et al. 2006, Bowden et al. 2006, Chen et al. 1985, Chen, 1989, Okamura and Resh 1995, Osiak et al. 2005, Varon et al. 2006
I–III	SSeCKs	Prevented podosome formation measured by colocalization of Tks5/Fish and actin in NIH3T3 cells expressing temperature-sensitive v-Src. Matrigel invasion was inhibited.	Gelman and Gao 2006
III–IV	TGFβ, SnoN	In breast cancer cells, absence of SnoN (shRNA) promoted TGFβ induction of holes formation by invadopodia; and degradation was inhibited by GM6001. TGFβ alone increased holes formation.	Zhu et al. 2007
I–IV	TGFβ/SMAD	Primary aortic endothelial cells form rosettes of invadopodia with associated MT1-MMP and requiring Src, PI3Kinase, and RhoA GTPase signaling. Collagen invasion was correlated with invadopodia and an extensive survey of colocalizing podosome components was performed.	Varon et al. 2006
I–III	Tks5/FISH	PX domain was required for Tks5/FISH plus Src to activate localized degradation in fibroblasts and breast cancer cells. Expression of deleted PX domain Tks5 plus Src repressed actin core formation. Matrix degradation by invadopodia was diminished by siRNA targeting Tks5.	Abram et al. 2003, Courtneidge et al. 2005, Seals et al. 2005
III–IV	TNFα, VEGF	Subconfluent HUVEC form invadopodia with associated MT1-MMP localized at sites of degradation in response to cytokines. This required Src and RhoGTPase signaling.	Osiak et al. 2005
II–IV	uPAR	uPAR colocalized with actin cores and sites of localized degradation in VSMC.	Furmaniak-Kazmierczak et al. 2007
II–III	WAVE1	siRNA blocking WAVE1 did not affect actin initiation sites but blocked degradation by invadopodia in mammary carcinoma cells.	Yamaguchi et al. 2005

(*continued*)

Table 21.1 (continued)

Stage[a]	Component[b]	Evidence for role in invadopodia[c]	Citation
I–III	WIP	siRNA targeting WIP blocked actin core of invadopodia. WIP interaction with N-WASP but not with cortactin was required for actin core formation. Localized to invadopodia.	Yamaguchi et al. 2005
I–III	α3β1, α6β1, integrin β1	In melanoma cells, activation of α6β1 integrin by laminin peptides induced association of FAP/seprase with integrin, and increased invadopodia-mediated degradation. Integrin, FAP/seprase, and uPA were associated at the cell surface of melanoma cells.	Artym et al. 2002, Mueller et al. 1999, Mueller and Chen 1991, Nakahara et al. 1996, 1998
II–IV	α4β1 integrin	α4β1 integrin and CXCR4 upregulate MMP-9. Integrin signaling regulated its localization and activity in podosomes of B-cell chronic lymphocytic leukemia and HUVEC cells.	Redondo-Munoz et al. 2006
	αvβ3 and β1 integrins	Associated with MT1-MMP in ovarian carcinoma cells and endothelial cells.	Deryugina et al. 2001, Ellerbroek et al. 2001, Galvez et al. 2002
	αvβ3 integrin	Associated with MT1-MMP and MMP-2 at the cell surface.	Brooks et al. 1996, Galvez et al. 2002

[a] Stage refers to Stages I–IV defined in Artym et al. (2006) and is inferred by comparison to cortactin/F-actin and MT1-MMP colocalization, and the degree of matrix degradation at the invadopodia:

Stage I: Initiation (cortactin/actin present)
Stage II: Preinvadopodia (cortactin/actin + MT1-MMP)
Stage III: Mature invadopodia (cortactin/actin + MT1-MMP + foci of matrix degradation)
Stage IV: Late invadopodia (MT1-MMP + degraded matrix).

[b] Invadopodia-related molecule.

[c] Evidence of colocalization with ECM-degrading proteases such as MT1-MMP or localized degradation in a fluorescent gelatin cross-linked matrix.

Proteolytic Activity of Invadopodia

Invadopodia are operationally defined as possessing membrane-associated proteolytic activity. Protease activity is localized to discrete sites that can be detected by small holes formed in a fluorescent matrix beneath the cell using microscopic imaging in the presence versus the absence of protease inhibitors or siRNA knockdown of proteases (Artym et al. 2006, Bowden et al. 2001, Chen et al. 1984, Kelly et al. 1994, 1998). The rationale for this assay is that membrane association of proteases limits proteolysis to sites of membrane contact. In the assay for localized degradation by invadopodia, small holes are visualized by high resolution

microscopic imaging since the holes are initially about 0.5 μm in size. The gelatin layer is either directly labeled with fluorescence or fluorescent fibronectin is bound to the unlabelled gelatin via its gelatin-binding domain. Assays are conducted in the presence of serum because, for the most part, invadopodia fail to degrade the matrix in the absence of serum factors (Mueller et al. unpublished data). Distinctive actin cores are formed in serum-free conditions in the presence but not in the absence of EGF (Yamaguchi et al. 2005). Other ECM components can be coupled to the matrix covalently and are similarly degraded by invadopodia (Kelly et al. 1994).

The term invadopodial complex has been used to define a subset of actin/cortactin-containing aggregates at the membrane that contain cortactin colocalizing with high levels of tyrosine-phosphorylated proteins (Bowden et al. 2006). This particular combination singularly identifies invadopodia that are active in matrix proteolysis since using the fluorescent-gelatin degradation assay, within individual cells, aggregates of cortactin/phosphotyrosine colocalize at sites of localized degradation (dark holes in the fluorescent layer), and comparison of cell lines reveals a strong correlation between numbers of aggregates of cortactin/phosphotyrosine and numbers of holes formed beneath cells. In addition to cortactin, others have reported a variety of invadopodia-related proteins such as Tsk5/FISH that colocalize with the center of the degradation hole and the actin core (Table 21.1).

MT1-MMP is one marker of invadopodia, particularly when it is colocalized with cortactin. It is required for invadopodia-mediated matrix degradation of cross-linked gelatin films (Artym et al. 2006), Table 21.1), it degrades collagen, it is required for invasion through collagen matrices, and it is a major enzyme involved in remodeling ECM during development and pathology (Itoh and Seiki 2006). TIMP-2, but not TIMP-1, and GM6001, or siRNA knockdown of MT1-MMP block invadopodia mediated degradation of 2D fluorescent gelatin films as well as collagen invasion in 3D systems (Artym et al. 2006, d'Ortho et al. 1998, Hotary et al. 2000, 2002, 2006; Sabeh et al. 2004, Varon et al. 2006, Wolf et al. 2007). The presence of MT1-MMP aggregates may not always be associated with proteolysis (Wolf et al. 2007), and thus its arrival at invadopodia might precede maturation and activation. However, there is also uncertainty as to when matrix proteolysis truly begins since visualization of the formation of a hole or the appearance of dequenched, proteolyzed DQ-collagen has a finite sensitivity. And, MT1-MMP is not the only enzyme that localizes to invadopodia and participates in invasion; others are present, for example, uPAR, FAP/seprase, MMP-2, MMP-9, and ADAM-12 [Table 21.1 (Linder 2007)]. The target substrates for these additional proteases and their function during invasion vis-a-vis invadopodia are not fully appreciated.

Actin/Cortactin Cores

The simple presence of cortactin/actin aggregates or "cores" at the membrane does not necessarily indicate proteolytic activity and thus, they do not identify mature invadopodia (Artym et al. 2006, Bowden et al. 2006). In the absence of proteolysis,

visualization of a cortactin/actin core might occur before the invadopodia has matured and acquired MT1-MMP and proteolytic activity [Fig. 21.1 (Artym et al. 2006)]. Alternatively, cortactin/actin aggregates might represent other structures unrelated to invadopodia or with an alternate function such as endocytic vesicles (Cosen-Binker and Kapus 2006, Kaksonen et al. 2000).

Podosomes of monocytic cells and v-Src transformed fibroblasts are often defined by F-actin cores surrounded by a ring of vinculin (Buccione et al. 2004, Linder, 2007). Similarly, the actin cores of invadopodia often have a distinctive appearance as do the resulting pattern of holes formed in the fluorescent matrix (Bowden et al. 1999, 2006, Linder, 2007, Weaver 2006). In the case of v-Src transformed chicken embryo fibroblasts, these podosomes may be collections of invadopodia with matrix-degrading capability on matrix (Chen, 1989). The ring shape that contains focal adhesion proteins vinculin, α-actinin, paxillin, and tensin serves to distinguish podosomes from focal adhesions which have a linear, elliptical shape. The presence of the vinculin ring has been reported in podosomes of transformed fibroblasts, smooth muscle cells, endothelial cells, and activated blood cells such as macrophages (Linder and Aepfelbacher 2003). Vinculin associated with podosomes can take several forms, a simple ring around each individual podosome (Marchisio et al. 1987) or associated with clusters of podosomes to produce a ribbon-like pattern of vinculin staining (Schliwa et al. 1984).

However, vinculin rings appear to be absent in breast cancer cells forming invadopodia (Artym et al. 2006) and eosinophils (Johansson et al. 2004). Since vinculin is associated with adhesions, the presence or absence of vinculin surrounding actin cores might relate to the diversity of integrin-mediated adhesions in different cell types or on different matrices. For example, a potential collaboration between α5β1 integrin and α3β1 integrin in melanoma cells was suggested by the close proximity of their staining patterns, α3β1 integrin colocalized with the hole at the site of degradation and α5β1 integrin in areas surrounding the site of degradation (Mueller et al. 1999). The α5β1 integrin adhesions were suggested to be focal adhesion-like.

Ultimately, high resolution imaging and related techniques must be used to sort out the molecular associations and structures that comprise the invadopodia and closely associated adhesive sites at the leading edge of invasive cells; this is a prerequisite to determining the mechanisms of cellular invasion. However, a ring of vinculin, although it might be diagnostic for podosomes in some cell types, is not a useful identifier for tumor cell invadopodia.

Stages of Invadopodia Formation and Acquisition of Proteolytic activity

The formation of invadopodia can be elucidated using time lapse imaging and multicolor immunofluorescence to identify the actin core components or proteases together with the functional proteolytic activity of mature invadopodia (Artym et al.

2006, Lorenz et al. 2004). Thus, de novo formation of invadopodia can be studied using confocal microscopy of samples fixed at increasing times after initially seeding cells on a fluorescent matrix (gelatin). Pairs of invadopodial components including cortactin, pY, Tks5/FISH, and MT1-MMP together with phalloidin staining to detect F-actin and fluorescent matrices to detect localized degradation can be used to determine the relative prevalence of each with time after seeding cells. Invadopodia begin forming within 30 min after seeding cells on the fluorescent matrix, depending on the cell type. The preponderance of cortactin/actin cores, cortactin/MT1-MMP, or cortactin/MT1-MMP/holes with time after seeding cells was used to infer the existence of various stages of invadopodia formation (Artym et al. 2006).

Using the more accurate live cell, time lapse imaging approach, Artym et al. (2006) found that the time span between the arrival of cortactin at invadopodia initiation sites and the arrival of MT1-MMP and detection of the hole forming in the matrix is on the order of minutes. Relative to cortactin, an actin-binding protein, MT1-MMP, an ECM-degrading enzyme, and detection of localized degradation of the fluorescent matrix, four stages of invadopodia formation were characterized: initiation, preinvadopodia, mature invadopodia, and late invadopodia (Artym et al. 2006). Initiation (stage I in this model) is marked by the appearance of cortactin aggregates at the membrane. Formation of preinvadopodia is indicated by the appearance of MT1-MMP aggregates colocalizing with the cortactin aggregates (stage II). Maturation of invadopodia begins with the appearance of a hole in the fluorescent matrix just under the aggregates of cortactin and MT1-MMP (stage III), and full maturation of invadopodia is signaled by the disappearance of cortactin from the site leaving the MT1-MMP aggregate behind (stage IV). Figure 21.2 illustrates these stages in a time line of invadopodia formation.

Studies which have connected the presence and signaling pathways of invadopodia or podosomes to the activity of localized degradation of a gelatin matrix are listed in Table 21.1. The original description of these stages relied on the knowledge that at a given cortactin/actin core appearing at the cell membrane, a hole or site of localized degradation would or would not eventually form underneath it. Thus, each stage has a specific criterion for membership including the eventual formation of a hole, and molecular membership of invadopodia components. Invadopodia proteins were hypothetically assigned to stages based on whether cortactin/actin, MT1-MMP, or proteolytic degradation were simultaneously detected in the study (Table 21.1).

Invadopodia dynamics can be followed by measuring the time that elapses between the appearances of each stage (Artym et al. 2006). The delays between cortactin accumulation, MT1-MMP accumulation, and initiation of matrix degradation were different for migrating versus nonmigrating breast cancer cells suggesting cross talk during cell migration and membrane-associated matrix degradation (Artym et al. 2006). Invadopodia are also dynamic from a molecular point of view. Baldassarre et al. (2006) reported actin comets associated with sites of degradation, and perhaps corresponding to this activity, other investigators have shown highly dynamic exchange of actin at podosomes and invadopodia (Destaing et al. 2003).

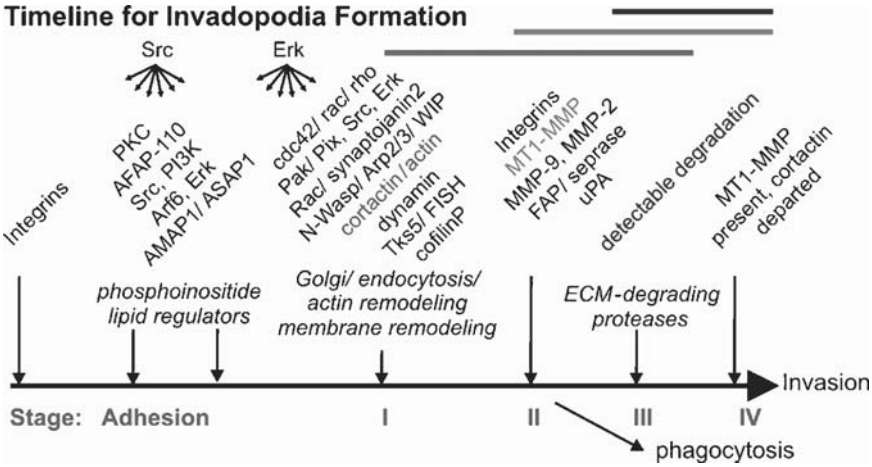

Fig. 21.2 Time line for invadopodia formation. The stages of invadopodia are presented along a time line and prominent invadopodia-associated proteins are hypothetically assigned to positions along the time line according to studies colocalizing them with cortactin/actin, MT1-MMP, or gelatin degradation. In color, the template for the time line is illustrated by cortactin/actin (*red*), MT1-MMP (*green*), and detectable degradation (*blue*) (*See also* Color Insert I)

Cortactin was associated with retrograde actin flow in NIH 3T3 cells and vesicles appear to be propelled by comets of actin and cortactin (Kaksonen et al. 2000). Yamaguchi et al. have characterized invadopodia visualized using GFP-actin. Actin cores appeared to be motile over the membrane and their motility was inversely related to the intensity, that is, actin content (Yamaguchi et al. 2005). They found that the longer lived cores tended to stay in one spot and were even left behind as cells migrated away.

In summary, invadopodia, like podosomes, are highly dynamic as demonstrated using time lapse imaging (*see* Artym et al. 2006, Ayala et al. 2006, Baldassarre et al. 2003, Evans et al. 2003, Linder, 2007, Linder and Aepfelbacher 2003, Yamaguchi et al. 2005 for discussion and review).

Relating Functional Components to Stages of Invadopodia

In reviewing published studies, those invadopodia/podosomes that were described by characterization of the presence or absence of actin/cortactin cores before and after drug treatment, mutant protein expression, or siRNA knockdown, but containing no investigation of localized matrix degradation, could tentatively be termed Stage I invadopodia (cortactin/actin localization, no MT1-MMP, no hole formed). However, if the study correlated the presence or absence of cortactin/actin with MT1-MMP, a tentative assignment to Stages II–III might also be made. Similarly, if presence or absence of holes was simultaneously determined, then the drug, siRNA, or mutant protein effect could be attributed to effects on Stages I–IV.

Finally, if cortactin/actin, MT1-MMP, and holes were simultaneously investigated, then the outcome of each experiment could be understood more fully in terms of the time course of invadopodia formation and maturation that was characterized in breast cancer cell lines. Table 21.1 represents the results of a review conducted in this fashion. The molecular classes of invadopodia components have been integrated into the time course of invadopodia formation based on the molecular identification and functional studies associated with each report in the literature (Figs. 21.1 and 21.2, Table 21.1). For comparison some other molecules for which studies suggest a similar assignment are depicted in Fig. 21.2.

Invadopodia can have long- or short-term life spans, from minutes to hours (Yamaguchi et al. 2005). Since only GFP-actin was monitored, we presume these invadopodia to be members of a stage between I and III (Fig. 21.2). This was determined by comparison with the Src-activated MDA-MB-231 cells in which these stages were originally defined. In the latter case, presence of cortactin aggregates was tightly correlated with presence of F-actin aggregates suggesting their interdependency (Artym et al. 2006). Yamaguchi et al. (2005) suggest that invadopodia arise and operate in three stages: initiation, searching, and maturation. Time lapse imaging indicates that the short-lived invadopodia may be a precursor to the long-lived, immobile ones and that these two groups could be, respectively, thought of as a "searching" and "maturation" phase. The difference between the two relates to changes in strength of adhesion to the matrix. One possibility is that searching and maturation are equivalent to Stages I–III (Stage IV lacks cortactin/phalloidin-bound F-actin), where the difference in adhesion relates to the arrival of MT1-MMP and other associated proteins. Exploring further the dynamics of the EGF-induced invadopodia, they found that the lifetimes of the invadopodia were affected by cofilin knockdown (Yamaguchi et al. 2005) with the consequence that the longer lived population of invadopodia was lost. Consequently, degradation was reduced, although not eliminated. This suggests that, depending on the cell type, invadopodia lifetimes may reflect differences in prominence of signaling pathways, particularly cofilin-signaling pathways. And, it suggests that long-lived invadopodia might be equivalent to Stage III and contributing most heavily to matrix degradation, whereas short-lived invadopodia are equivalent to Stages I and II.

Invadopodia and Their Relationship with Pseudopodia and Cellular Protrusions in 3D

Returning to the discussion of invadopodia as forming on the tips of pseudopodia or pseudopodial branches, examination of cortactin and MT1-MMP localization together with protease activity in 3D cultures is the next logical step to relating 2D studies to in vivo formation and function of invadopodia. In thicker matrices with high rigidity, such as cross-linked gelatin layers, it appears that the cell migrates to "follow" the initial invadopodia membrane that has penetrated the matrix in advance, engulfing partially degraded matrix (Fig. 21.1, compare a, *iii* and a, *iv*) (Baldassarre et al. 2003,

Coopman et al. 1996, Lorenz et al. 2004, Mueller and Chen 1991). However, cortactin and MT1-MMP have not been colocalized in these settings.

However, in studies of tumor cells invading through a 3D collagen lattice, MT1-MMP appears punctate over much of the cell surface, suggesting that formation of pseudopodia and their branch formation was minimal (Wolf et al. 2007). However, under these conditions, cells formed linear tracks of advancement, with cell–to–cell interactions occurring along their lengths. Evidence of protease-mediated collagen degradation was obtained for a region of the leading cell subtending the leading edge and thus probably associated with only a subset of the MT1-MMP punctae (Wolf et al. 2007). It is interesting to compare this 3D study with that of the 2D study of Artym et al. (2006) since hypothetically, the MT1-MMP punctae at the leading edge of the cell in the collagen lattice might represent the newest sites of invadopodia formation, that is, Stage II (Fig. 21.2a, *ii*), and those more rearward in the cell might represent invadopodia that have acquired proteolytic activity, that is, Stage III (Fig. 21.2a, *iii*), and then later maintained proteolytic activity but lost cortactin, that is, Stage IV (Fig. 21.2a, *iv*). Confirmation of this could be obtained by staining both cortactin and MT1-MMP.

Functional Consequence of Invadopodia-Mediated Degradation

Proteases Mediate Matrix Degradation as well as Stimulate Cross-Talk Between Cells and the Extracellular Environment

Matrix degradation is perhaps the best known function of invadopodia. Indeed they are distinguished from focal adhesions by their matrix-degrading capability and their more transient existence (Chen et al. 1984, Chen 1989). Invadopodia are capable of degrading a wide range of ECM molecules including fibronectin, laminin, type-I collagen, as well as glutaraldehyde cross-linked gelatin (Kelly et al. 1994, Mueller and Chen 1991). It is evident that increased activities of a variety of proteases must occur at invadopodia. Early work indicated that a 170 kDa serine protease now known as seprase or fibroblast activation protein-α (FAP) and MMP-2 were associated with invadopodia (Aoyama and Chen 1990, Emonard et al. 1992, Monsky et al. 1993). There are now at least eight proteases known to localize to invadopodia (Chen et al. 2003, Ghersi et al. 2003, Linder 2007, Weaver 2006). Importantly, concentration to invadopodia is critical for the heightened proteolytic activity. Integrins are key players in ligating proteases to invadopodia. For example, FAP association with $\alpha_3\beta_1$ integrin that is stimulated by collagen is well documented (Mueller et al. 1999) as is the increased surface localization and concentration to invadopodia of FAP when $\alpha_6\beta_1$ integrin is ligated with laminin peptides (Nakahara et al. 1996). In addition to binding $\alpha_3\beta_1$ integrin, FAP also ligates other proteases to invadopodia through its ability to form larger complexes with the related serine protease dipeptidyl peptidase IV (DPPIV) (Ghersi et al. 2002, 2006). The close association of FAP with urokinase-type

plasminogen activator receptor (uPAR) in the plasma membrane of aggressive melanoma cells is also mediated by β_1 integrins (Artym et al. 2002). Indeed, $\alpha_3\beta_1$ integrin that is known to bind to FAP (Mueller et al. 1999) also binds to uPAR (Zhang et al. 2003). Thus, FAP, DPPIV, and uPAR are organized in invadopodia through binding interactions that hinge around $\alpha_3\beta_1$ integrin and FAP.

MT1-MMP forms functionally important complexes with MMP-2 at invadopodia (Nakahara et al. 1997) that likely cooperate with FAP–DPPIV–uPAR–integrin complexes to efficiently degrade ECM (Kelly 2005). The active form of MMP-2 was the first MMP shown to localize to invadopodia (Emonard et al. 1992, Monsky et al. 1993). More recently, MMP-9 has been identified in podosomes of B-chronic lymphocytic leukemia cells and to have an important role in transmigration through endothelial cell barriers (Redondo-Munoz et al. 2006). MMP-mediated proteolysis is critical for the matrix degrading and invasive functions of invadopodia (Kelly et al. 1998). The molecule that binds MT1-MMP to invadopodia is not known, but the linkage involves the transmembrane domain of MT1-MMP (Nakahara et al. 1997) and the intact invadopodia cytoskeleton that is formed only when cortactin is present (Artym et al. 2006). Evidence has suggested a possible interaction between $\alpha_v\beta_3$ integrin and the MT1-MMP, MMP-2 complexes (Brooks et al. 1996, Deryugina et al. 2001). Not only are integrins involved in localizing MMPs, but they are also often involved in upregulating their expression (e.g., Lochter et al. 1999).

Together FAP/seprase, DPPIV, uPA, MT1-MMP, MMP-2, and MMP-9 account for six of the eight proteases known to reside in invadopodia. The other two are a disintegrin and metalloprotease-12 (ADAM-12) that has been found in the podosomes of Src-transformed cells (Abram et al. 2003) and a distinct type of metalloproteinase called invadolysin initially discovered in *Drosophila melanogaster* mutants that produced aberrant mitotic spindles (McHugh et al. 2004). The roles of these enzymes in invadopodia are not known but they may contribute to other functions of invadopodia such as modification of chemokines and cytokines.

These eight proteases that localize to invadopodia may not reflect the entire armament of invadopodial proteases because there may be others that have not been investigated. However, between them they can degrade most ECM molecules. Moreover, uPAR presumably concentrates uPA to invadopodia where it can activate plasmin in extracellular fluids. Plasmin degrades multiple ECM substrates and activates MMPs that degrade a broad spectrum of matrix molecules. Thus, invadopodia are equipped with all the proteases needed to break down matrix barriers and promote cell invasion. But, eroding matrix barriers is only one function of the proteases at invadopodia. They also participate in the ongoing "conversation" between motile cells, the ECM, and the cells present in the microenvironment.

Invadopodial Proteases and the Tumor Microenvironment

The proteolytic activity of DPPIV is known to have roles in blood glucose regulation, leukocyte migration, and angiogenesis through cleavage of glucagon-like peptide and glucose-dependent insulinotropic polypeptide; CXCL12 and CCL22

chemokines; and neuropeptide-Y (Chen et al. 2003). In this way, invadopodial proteases can serve as cellular traffic directors modulating the response of cells surrounding invading cells that express invadopodia.

FAP appears to have multiple roles in tumor—host interactions. For example, overexpression of FAP on tumor cells stimulates angiogenesis and rapid tumor growth in an animal model of human breast cancer (Huang et al. 2004). Moreover, FAP was identified in a homology cloning approach as important for capillary morphogenesis and angiogenesis of microvascular endothelial cells (Aimes et al. 2003). FAP–DPPIV complexes are found on microvascular endothelial cells and their action is required for endothelial cell invasion of collagen (Ghersi et al. 2006). Thus, FAP apparently has a direct role in communications between tumor cells and the extracellular environment to stimulate the angiogenesis that sustains rapid tumor growth. The pro-angiogenic function of FAP may be enhanced by MMP-9. MMP-9 has been implicated in proangiogenic functions through release of matrix-bound vascular endothelial growth factor (Bergers et al. 2000, Vu et al. 1998). However, MMP-9 also has antiangiogenic functions through the production of tumstatin, an antiangiogenic fragment derived from MMP-9 cleavage of type-IV collagen (Hamano et al. 2003). More evidence for potential cooperation between FAP and MMP-9 comes from the recent finding that FAP and MMP-9 produce distinct but comparable low-molecular weight fragments of denatured type-I collagen (Christiansen et al. 2007). Moreover, new work has shown that recombinant and soluble FAP activity can be increased up to sevenfold by truncation of FAP by EDTA-sensitive proteases (Chen et al. 2006). Indeed, this elevated activity of FAP is seen in tumors of ovarian cancer (Chen et al. 2006) and breast cancer (Kelly 1999). While the identities of the EDTA-sensitive proteases that mediate the hyperactivation of FAP are unknown, it is tempting to speculate that they are MMPs such as MMP-9. Although recombinant soluble FAP was used to detect the phenomenon of hyperactivation of FAP, there is now evidence that a soluble form of FAP occurs naturally in blood (Lee et al. 2006b).

McKee's group has identified a soluble form of FAP in the plasma as the α_2-antiplasmin cleaving enzyme (APCE) (Lee et al. 2006b). FAP cleaves the N-terminal 12 amino acids from α_2-antiplasmin, rendering it 13 times more capable of penetrating fibrin aggregates (Lee et al. 2006b). α_2-antiplasmin inhibits plasmin, the major fibrinolytic enzyme in serum, making it possible that overexpression of FAP observed in melanoma, breast, and many epithelial cancers could directly contribute to stabilizing fibrin, making fibrin microclots and microemboli more likely in these cancer patients.

In summary, invadopodial proteases are complexed together on the surface of the plasma membrane. These complexes are both adhesive and lytic. They are sites of cell signaling because integrins are included in the complexes but they are also sites where cells proteolytically modify the matrix. These modifications not only facilitate migration of cells through matrix barriers, they are also avenues for cell–cell communication. Through the actions of invadopodial proteases, growth and angiogenic factors are released from the matrix, cytokine functions are altered, and bioactive fragments of matrix molecules are produced. These evoke biological

responses from surrounding cells. In the case of malignant tumors the result of these responses is the angiogenesis that fuels rapid tumor growth. Thus, the invadopodial proteases, particularly FAP and DPPIV, are appealing targets for therapeutic interventions (Kelly 2005). Future work will determine the roles of proteolytic activities at invadopodia and those functions mediated by complex formation and subsequent signaling.

Various reports suggest that invadopodia-like protrusions might also be involved in a variety of processes, for example, diapedesis (Carman et al. 2007), eosinophils adhering to VCAM-1 via podosomes (Johansson et al. 2004), anchor cell invasion through basement membrane in *Caenorhabditis* elegans (Sherwood et al. 2005), and migration of border cells during Drosophila development (Fulga and Rorth 2002). In one of these cases, degradation of the cell–cell adhesion molecule VCAM-1 required the podosome-associated disintegrin metalloprotease ADAM-8 (Johansson et al. 2004). Thus, invadopodia might serve other functions in addition to ECM degradation and might participate in a wide array of developmental and normal physiologic processes in addition to tumor cell invasion. Furthermore, invadopodia might possess more than passing resemblance to growth cone filopodia, for example, and opportunities for direct comparison could shed light on the variety of functions that these most interesting cellular structures mediate.

Conclusions and Future Perspective

Moving forward, clarification of the relationship between podosomes and invadopodia will require careful comparisons between cell types, between cells on defined matrices, and using multiple imaging approaches including time lapse imaging. Previously, studies of podosomes were conducted on glass substrata, whereas studies of invadopodia were performed on matrix-coated substrata, by necessity, in order to detect localized matrix degradation. The ultimate challenge, in the future, will be to detect and study the role of invadopodia in living tissues and to identify their association and participation in pathological cell invasion in vivo. The study of podosomes and invadopodia using advanced imaging techniques in model systems, and within 3D settings in vitro and in vivo is just taking off and is facilitated by the advent of new high-resolution imaging capabilities including multiphoton imaging, multispectral imaging, fluorescence detection of protease activity in vivo, second harmonic visualization of collagen fibers, antibodies to detect cleaved collagen, fluorescence resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), and the development of siRNA-knockdown techniques to catalogue required molecular components, structures, molecular interactions, and functional consequences of invadopodia formation [(Artym et al. 2006, Destaing et al. 2003, Evans et al. 2003, Lorenz et al. 2004, Wolf et al. 2007) and references therein].

Thus, major questions in invadopodia research include determining the functional relationship between podosomes and invadopodia, understanding structural and functional variations from cell type to cell type; identifying exact placement of

each invadopodia-related molecule in the time course of invadopodia and then determining their functional roles and molecular interdependencies. Ultimately, the goal is to determine the in vivo relevance of invadopodia and podosomes for disease progression, and to discover how they might be therapeutically targeted to control pathological cell invasion during tumor formation and metastasis.

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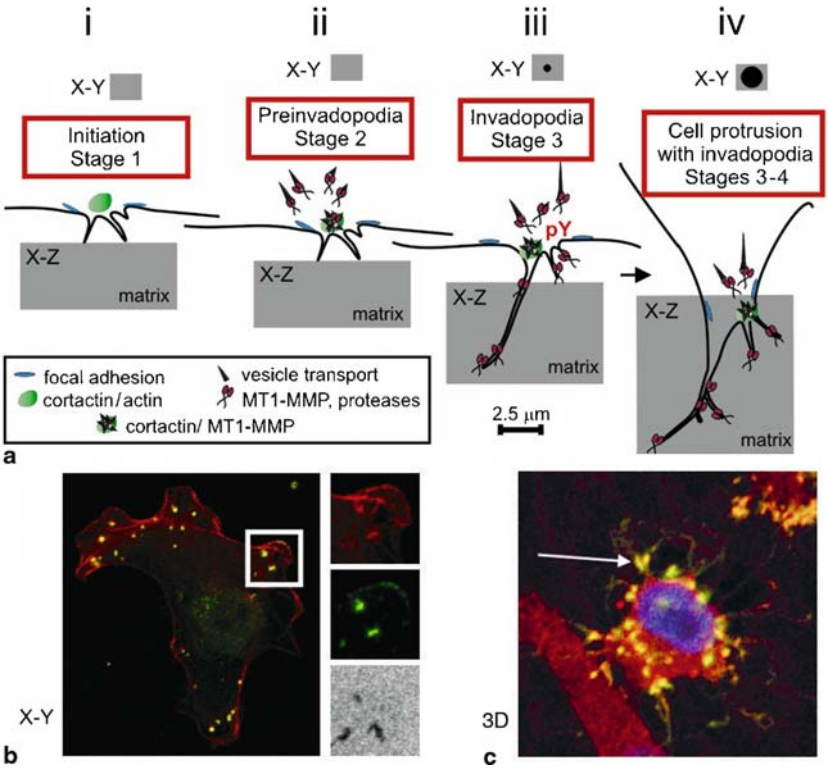

Fig. 21.1 Stages of invadopodia in 2D and 3D culture settings. **a** *i–iv* illustrate the initial formation of invadopodia beginning with cortactin recruitment (**i**), followed by recruitment of proteases such as MT1-MMP (**ii**), extension of invadopodia membrane into matrix facilitated by membrane-associated proteases and (**iii**), the enlargement of the site of degradation and invasion as the cell extends a pseudopodia/protrusion containing invadopodia and adhesion sites. **b** MDA-MB-231/c-Src(Y527F) cells breast cancer cells were immunostained using anticortactin 4F11 mAb (*green*) and counterstained with phalloidin (*red*) and imaged using confocal microscopy. The gelatin matrix (*grey*) contained sites of degradation and colocalizing F-actin and cortactin (*contained in insets*). **c** MDA-MB-231/c-Src(Y527F) cells were cultured on a thicker gelatin matrix whose autofluorescence is visualized together with cortactin in the red channel, DAPI in the blue channel, and antiphosphotyrosine 4G10 mAb staining in the green channel of this confocal image. Invadopodia are seen projecting into the matrix around the cell and aggregates containing colocalized cortactin and phosphotyrosine are visualized as yellow dots (*arrow*)

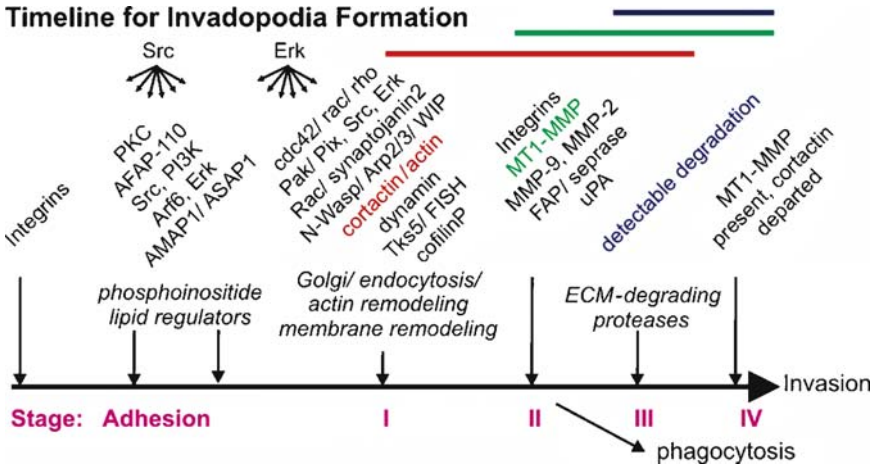

Fig. 21.2 Time line for invadopodia formation. The stages of invadopodia are presented along a time line and prominent invadopodia-associated proteins are hypothetically assigned to positions along the time line according to studies colocalizing them with cortactin/actin, MT1-MMP, or gelatin degradation. In color, the template for the time line is illustrated by cortactin/actin (*red*), MT1-MMP (*green*), and detectable degradation (*blue*)