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The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function

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PREFACE

Podosomes and invadopodia are actin-based dynamic protrusions of the plasma membrane of metazoan cells that represent sites of attachment to, and degradation of, the extracellular matrix. Key proteins in these structures include the actin regulators cortactin and (N)-WASP, the adaptor proteins Tks4 and Tks5, and the metalloprotease MT1-MMP. Many cell types elaborate these structures, including invasive cancer cells, vascular smooth muscle and endothelial cells, and immune cells such as macrophages and dendritic cells. Recent progress has been made in our understanding of the regulatory and functional aspects of podosome and invadopodia biology and their role in human disease.

INTRODUCTION

In 1980, David-Pfeuty and Singer demonstrated that transformation of chicken embryo fibroblasts with Rous sarcoma virus (RSV), containing the oncogene *src*, caused relocalization of the cytoskeletal proteins vinculin and α-actinin away from the cell-extracellular matrix (ECM) contact points called focal adhesions and into circular clusters they called **rosettes**[1]. In 1985, Tarone, Marchisio and their colleagues demonstrated that these proteins were localized to protrusions of the ventral membrane that also contained actin and tyrosine phosphorylated proteins, and were sites of cell adhesion to the extracellular matrix[2] (Box 1). They considered these structures cellular feet, and therefore called them **podosomes**. That same year, and also using RSV-transformed cells, Chen, Parsons and colleagues demonstrated that the tyrosine kinase Src was localized to sites of cell contact with the extracellular matrix[3] (Box 1). Furthermore, they made the important observation that degradation of the ECM occurs at these contact sites[3]. In 1989, Chen demonstrated that these Src-enriched sites of degradation were in fact the podosomes[4]. To reflect the adhesive and degradative capacity of these structures, Chen coined the term **invadopodia**. Chen and colleagues subsequently extended these findings beyond RSV-transformed chicken and mouse fibroblasts to show that invadopodia could also be found in human cancer cell lines. In the meantime, Marchisio and colleagues demonstrated that podosomes could form in cultured osteoclasts[5]. They have now been described in other cell types, including macrophages and dendritic cells, endothelial cells and vascular smooth muscle cells[6]. The past twenty- five years has seen an expansion on research into podosomes and invadopodia, including the discovery of associated proteins, of stimuli required for their formation, and of in vivo relevance (Box 1 and Table 1). However, the control of their formation, and their function in vivo, remains somewhat enigmatic.

Given that the terms podosome and invadopodia were first applied to the same structure in the same cells — the ventral protrusions of Src-transformed fibroblasts — it is hardly

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surprising that confusion surrounds the nomenclature! Typically, structures of this type are referred to as podosomes when found in normal cells, and invadopodia when found in cancer cells. But what about the Src-transformed cells where they were originally discovered? Some of us have used podosomes and invadopodia interchangeably in this case, no doubt adding to the confusion. However, these cells are truly cancer cells, faithfully mimicking all the tumorigenic properties of the fibrosarcomas caused by RSV in its native host. Therefore, going forward, we advise using the term invadopodia to describe Src-transformed fibroblasts, but recommend using both search terms, and noting the cell type under study, when evaluating all older literature. A final comment on nomenclature: most recently a catch-all term — the **invadosome** — has been introduced to describe all adhesive structures involved in ECM degradation and invasion. This term can be convenient where no distinction is being made between podosomes and invadopodia. However, it would seem premature to replace the original terms until and unless it becomes clear that podosomes and invadopodia are identical. In this Review, we will use the 'normal cells = podosomes' and 'cancer cells = invadopodia' rule.

Our focus in this Review is on recent progress in understanding how podosome and invadopodia formation and function are regulated, and how these structures impact development and disease. We will discuss individual podosome and invadopodia components in this context.

THE CHARACTERISTICS OF PODOSOMES AND INVADOPODIA

Both podosomes and invadopodia are characteristically composed of an actin-rich core surrounded by adhesion and scaffolding proteins[7]. A chorus of actin nucleators, polymerization activators, actin binding and cross-linking proteins, kinases, small GTPases and scaffold proteins regulate the actin machinery within these dynamic structures[6], with the half-life of actin turnover ranging from minutes to a few hours. Key players include the adaptor proteins Tks4 and Tks5, the actin regulators cortactin and (N)-WASP, the tyrosine kinase Src, and the transmembrane metalloprotease MT1-MMP. Some of these proteins are shown in Figure 1, but readers are referred to other recent reviews for comprehensive descriptions of all the molecular components of podosomes and invadopodia[6,8–10].

Are podosomes and invadopodia in fact distinct structures? Here, opinion differs. Some take the view that they vary in both structure and function. Others consider that there is no precedent for cancer cells "inventing" new mechanisms (rather than co-opting and dysregulating normal cellular processes) to argue that they are, in essence, identical structures. Nevertheless, while podosomes and invadopodia are very similar in overall architecture and function, morphological and molecular distinctions have been noted (Table 1). For example, previous studies have suggested that invadopodia protrude further into the ECM and are stable for hours, when compared to the minimal protrusion and rapid turnover of podosomes, and that this accounts for the higher degradative ability of cancer cells[11]. More recently, total internal reflection fluorescence (TIRF) microscopy studies suggest that invadopodia are part of a superstructure found in areas of membrane ruffling that is composed of a core with filament-like invadopodia emanating from it[12]. In contrast, podosomes neither exhibit intense membrane ruffling nor form filament-like processes[12]. However, it has recently been demonstrated that under appropriate culture conditions, dendritic cells can elaborate long podosomes into ECM and degrade[13]. How many of the perceived differences will be explained by the culture conditions, or the cell type being examined, remains to be determined.

It is also important to distinguish podosomes and invadopodia from other cellular protrusions such as filopodia and lamellipodia, and from other adhesive structures such as

focal adhesions. Each has distinct morphological characteristics (Table 1), and each form in a distinct spatial location in cells growing in 2D. Yet they share several common proteins, particularly those that orchestrate actin polymerization[14]. Therefore, it is necessary to consider protein and lipid composition, morphology and localization to distinguish between these structures (Table 1). In particular, the co-localization of ventral actin puncta with focal degradation of the ECM is a valuable means to distinguish podosomes and invadopodia from other membrane structures (Figure 2).

THE FUNCTION OF PODOSOMES AND INVADOPODIA

It is thought that podosomes or invadopodia allow a cell to coordinate ECM degradation with cell motility, to facilitate cell migration through tissue microenvironments. Cell migration is required during embryonic development and in adults in response to injury and infection. Abnormal cell migration can underlie developmental, vascular and immune diseases, as well as tumor metastasis. In keeping with this, podosomes are found in cell types involved in tissue remodeling and immune surveillance, and the presence of invadopodia is correlated with the ability of cancer cells to invade and metastasize

The maturation process for podosomes and invadopodia involves the recruitment and activation of multiple pericellular proteases, which facilitates ECM degradation[15], and perhaps also cytokine release (Table 1). There are three main classes of protease present in these structures: zinc-regulated metalloproteases (MMP2, MMP9, MT1-MMP, the ADAM family of sheddases), the cathepsin cysteine proteases, and the serine proteases seprase and urokinase plasminogen activator[16].

It is generally accepted that the invadopodia formed in human cancer cells and Src-transformed cells degrade ECM. However, there is some debate about the role of podosomes in ECM degradation. Earlier studies observed only shallow ECM degradation in podosome-containing cells[16], and degradative ability was once thought to distinguish invadopodia from podosomes[17]. However, more recent studies in vascular smooth muscle cells, dendritic cells, and endothelial cells have demonstrated degradation of ECM[13,18–21]. From these and other studies, it appears that ECM degradation by podosomes is likely to be dependent on cell type and ECM substrate. Perhaps podosomes might also play a degradation-independent role in cell migration.

How might podosomes and invadopodia control cell motility? Planar cell migration is facilitated by the coordination of focal adhesion assembly, maturation and turnover with protrusion of the leading edge at lamellipodia[22]. Collective cell migration requires MT1-MMP degradation of collagen to generate ECM tracks[23]. Additionally, mechanical forces are recognized to play an important role, both for the formation of focal adhesions, and for the growth of adhesion-anchored stress fibers. Recently, podosomes and invadopodia were also shown to act as mechanosensors, as well as to exert traction on the underlying matrix, in a process requiring MT1-MMP[24,25]. More insight is likely to be achieved through investigating the dynamics of the actin comet-based structures of invadopodia, which consist of a stationary head that is localized to degradative patches and a tail that is motile[26]. Interestingly, these structures disappear during cell migration. A detailed understanding of the mechanism by which podosomes and invadopodia facilitate cell migration is not yet available, and will not be covered here.

VISUALIZING PODOSOMES AND INVADOPODIA

We have introduced the concept that podosomes and invadopodia can be distinguished from other protrusive and adhesive structures by a careful analysis of their morphology and protein and lipid composition. Here, we will review their morphological characteristics.

Podosomes and invadopodia in 2 dimensions

Both podosomes and invadopodia are usually visualized by co-staining cells with fluorescent phalloidin, which binds to F-actin, an obligate component of these structures and other associated-proteins such as the actin nucleator Arp2/3 or cortactin, which promotes the polymerization and rearrangement of the actin cytoskeleton. Since these proteins also co-localize with actin in other cellular structures, it is important to determine that the co-staining is on the ventral surface of the cell, using either confocal or TIRF microscopy. As an alternative, the presence of both F-actin and the adaptor protein Tks5 can be diagnostic, since Tks5 does not appear to localize to other actin-based structures such as focal adhesions but is a key component of both podosomes and invadopodia[27–30]. It is particularly telling that the expression of Tks5 promotes the formation of invadopodia in cells that normally do not have them, in a manner dependent on its PX domain[31]. Finally, the co-localization of F-actin, cortactin or Tks5 with focal ECM degradation is often used as an identifier for podosomes and invadopodia.

In 2D culture, the formation of both podosomes and invadopodia is restricted to the ventral surface of the cells. The structures often present as isolated puncta, often behind the leading edge of the cell in the case of podosomes, or under the nucleus in the case of invadopodia (Figure 3). In some Src-transformed fibroblasts, individual invadopodia cluster together into rosettes[1] (Figure 3). Similar rosettes have also been observed in vascular smooth muscle cells, and occasionally in cancer cells. Rosette formation can be promoted by the expression of activated Src, activation of endogenous protein kinase C, Rho family GTPases, and certain integrins[21,32–37]. It is not known if there are functional differences between rosettes and individual podosomes or invadopodia. In osteoclasts cultured on glass, individual podosomes form transient circular rings that appear to fuse and form a podosome belt[38]. Under more physiological conditions, osteoclasts will form a similar F-actin rich structure composed of individual podosomes, called the sealing zone[39]. Perhaps clustering into rosettes and subsequent fusion is a maturation step that can occur in all cell types with the appropriate stimulus.

In 2D, podosomes are relatively short-lived (2 – 20 min) whereas invadopodia can persist for several hours[6]. Morphological study of cells with podosomes usually involves plating cells directly onto glass coverslips, while to study invadopodia, the coverslips are frequently coated with a layer of defined ECM. A systematic analysis of how these culture conditions might affect podosome and invadopodia number, size, distribution and turnover has not been undertaken, although it is known that increasing ECM rigidity increases invadopodia formation[40].

Podosomes and invadopodia in 3 dimensions

Cell culture in 3-dimensions (3D) is used to more faithfully mimic the *in vivo* environment and has been used to great effect by the research community to reveal key differences in the morphology, metabolism and survival of normal and cancer cells. Recent studies have used 3D systems to address podosome and invadopodia formation and function. In one of these, cancer cells were cultured on native basement membrane and the composition and function of invadopodia followed over time[41]. Passage of the cells through the basement membrane involved 3 stages that took place over 7 days: formation of invadopodia and perforation of the basement membrane at the sites of formation; extension and elongation of the invadopodia through and beyond the basement membrane; and invadopodia-led migration of the cells through the basement membrane[41]. Similar in depth studies of podosome formation in 3D have yet to be performed. However, when vascular smooth muscle cells and human monocyte derived macrophages are cultured in either collagen I or gelled collagen (fibrillar collagen with the architecture of Matrigel), respectively, they form long actin-rich

protrusions that contain podosome-associated proteins[42–44]. The extension of long podosome-like structures is also associated with robust degradation activity in human macrophages, dendritic cells, and lymphocytes[13,44,45]. Similar MMP-dependent protrusive structures have been seen in 3D cultures of Src-transformed mouse sarcomas, and human melanoma, fibrosarcoma and breast cancer cells[46–48]. That these 3D structures may represent podosomes and invadopodia is supported by the presence of F-actin together with proteins such as talin, cortactin, FAK, MT1-MMP, N-WASP, paxillin, gelsolin, and β1-integrin, although many of these markers are also found in other adhesive structures. It will be important to rigorously establish the characteristics that determine the existence of podosomes and invadopodia in 3D.

Podosomes and invadopodia *in vivo*

There is much circumstantial evidence to suggest that podosomes and invadopodia are physiologically relevant structures. For example, loss of the obligatory podosome proteins WASP and Src results in defects in macrophage and osteoclast function, respectively, *in vivo*[49,50]. And key invadopodia-associated proteins are required for cancer progression in animal models[51–53]. Yet there is currently scant evidence for the existence of invadopodia and podosomes *in vivo*, although we did recently visualize Tks5- and cortactin-containing rosette-like structures in vascular smooth muscle cells *in vivo*[19] (Figure 4). And our laboratory has also visualized Tks5-dependent protrusions in migrating embryonic cells (DAM and SAC unpublished) (Figure 4). With recent advances in the molecular characterization of the structures and enhanced microscopy techniques, we anticipate that the formation of podosomes and invadopodia will be a physiological event required for migration of many cell types *in vivo*.

PODOSOME AND INVADOPODIA INITIATION

Many studies have demonstrated that stimulation with growth factors such as PDGF, TGFβ, and EGF will induce podosome or invadopodia formation in normal and cancer cells, respectively[19,21,54,55]. These stimuli will elicit the phosphorylation and/or activation of key podosome and invadopodia proteins discussed above through canonical signaling pathways, especially those involving Src and PKC. Novel mechanisms for podosome and invadopodia initiation that have come to light more recently include ROS signaling, integrin signaling, and microRNA control. These will be discussed here.

Integrin signaling

Integrin receptors directly interact with the ECM and transmit extracellular signals to the cytoplasm (“outside-in signaling”), as well as the status of the cell to the extracellular space (“inside-out signaling’). They are thus ideally placed to modulate invadopodia and podosome biology. Many studies have described the localization of integrins to podosomes and invadopodia. For example, the αvβ3 integrin is found in both osteoclast podosomes and the invadopodia of several cancer types[56], and the β1 subunit is also found in both podosomes and invadopodia[57,58]. Few studies have directly addressed the role of the integrins in this structure, although podosome and invadopodia formation is modulated by the presence of ECM substrate[13,59]. Antibody-induced activation of β1 increases ECM degradation[60], while interference with αvβ3 in osteoclasts results in defective podosome function[61]. More recently, the roles of ECM and integrins in the regulation of podosome and invadopodia formation were investigated in osteoclasts and Src-transformed fibroblasts[36], which express both β1 and β3. Invadopodia still form in the absence of β3, but podosome and invadopodia formation is inhibited by loss of β1 (Figure 5)[59]. Furthermore, phosphorylation of the cytoplasmic tail of β1 promoted the formation of rosettes[59]. Interestingly, acute loss of β1 also promoted the formation of focal adhesions, reinforcing

the idea of a reciprocal relationship between these two structures, as described later (Figure 5).

Src kinases and PKCs

Src family kinases play a pivotal role in the formation of both podosomes and invadopodia. The scaffold protein Tks5 was first identified as a Src substrate, as were other proteins present in these structures, including cortactin, the actin binding and cross-linking protein AFAP110 and the integrin effector and adaptor protein p130Cas ([62,63]). Given this, it is perhaps not surprising that activated Src promotes podosome or invadopodia formation, whereas inhibition of Src has the opposite effect. Src stimulates primary actin nucleation, rate of flux at the actin core and formation of the actin cloud and podosome belt within osteoclasts, suggesting that one role is in the initiation of podosome formation[64]. But the observation that the podosomes that do form in Src−/− osteoclasts have a fourfold longer life span with decreased actin flux suggests that it is also required for the disassembly of podosomes[64]. How is Src activated in normal and cancer cells? Few studies have addressed this question directly, although both PDGF and EGF, which promote podosome and invadopodia formation, activate Src family kinases[65]. Src also mediates integrin signals to the actin cytoskeleton[66]. Src and PKC act in concert to regulate podosome and invadopodia formation[54,67]. For example, experimental activation of PKC-α with phorbol esters or by membrane targeting induces AFAP110 to co-localize with and activate Src, via a mechanism that requires PI 3-kinase activity[54]. Interestingly, PKC-mediated phosphorylation of AFAP110 and fascin, an actin bundling protein, stabilizes podosomes and invadopodia[46,68]. Thus PKC–Src mediated pathways appear to have a predominate role in podosome and invadopodia formation. Recent studies have identified novel Src-dependent regulatory mechanisms in podosome and invadopodia formation that involve integrins, reactive oxygen species, and microRNA.

Reactive oxygen species

Most reactive oxygen species (ROS) in the cell are produced either as a by-product of oxidative phosphorylation in mitochondria, or by the action of members of the NADPH oxidase (Nox) family. Nox-catalyzed ROS production occurs to high levels in phagocytic cells as a host defense mechanism, and at lower levels in other cell types to facilitate mitogenesis and motility[69]. In cancer cells, ROS are produced at high levels because of metabolic stress, resulting in DNA damage and apoptosis[70]. More recently it has become apparent that low level ROS production in cancer cells, likely from Nox enzymes, facilitates invasion and metastasis[70]. Both podosome and invadopodia formation are dependent on ROS, and Nox components and ROS generation were detected in invadopodia[28]. Interestingly, the podosome and invadopodia proteins Tks4 and Tks5 share some architectural similarity with the phagocytic Nox organizer protein p47phox, which promotes catalysis by recruiting p67phox and Rac GTPases to the membrane components of Nox2, via association with p22phox. In a similar way, Tks proteins can also associate with both p22phox and p67phox orthologues via reciprocal SH3 domain-polyproline motif interactions[28,71], which also promotes Nox catalysis. How do ROS promote invadopodia formation and function? Likely roles include increasing the expression of matrix metalloprotease (MMPs) and consequent remodeling of ECM, and transient modulation of the catalytic activity of key podosome and invadopodia regulators. For example, PKCs, and perhaps Src[72], are activated by ROS[73]. Furthermore, ROS can transiently oxidize and inhibit protein tyrosine phosphatases and some lipid phosphatases[74], which could also promote invadopodia formation. In keeping with this, knockdown of PTP-PEST increases invadopodia number[28]. In the future it will be important to catalog all phosphatases that are regulated by invadopodia-produced ROS, and determine their effect on invasiveness and migration.

MicroRNAs

MicroRNAs (miRs) are highly conserved small RNAs that inhibit gene expression, either by inhibiting translation or inducing degradation of target mRNAs, and in so doing control pathways involved in development, tumor growth and metastasis[75,76]. In vascular smooth muscle cells, miRs-143 and -145 regulate the switch between differentiated (contractile) and de-differentiated (synthetic or migratory) phenotypes, which occurs in response to vascular stress and injury[77,78], by controlling podosome formation both *in vitro* and *in vivo*[78,79]. The miRs normally repress podosome formation by inhibiting the expression of key podosome regulators PDGF receptor-α and PKCε (in the case of miR-143) and fascin (in the case of miR-145)[19]. Downregulation of the miRs, and subsequent podosome formation, is initiated by a pathway involving PDGF (which is released in response to vascular stress), Src and p53 ([19]). Currently it is not known whether select miRs also regulate invadopodia formation in cancer cells. However, there appears to be an overall down-regulation of miRs during transformation, metastasis, and metastatic relapse[75,80], suggesting that this is a possibility worth exploring.

ASSEMBLY AND TURNOVER

Podosomes and invadopodia are dynamic structures whose formation and turnover are tightly controlled. Here we will discuss what is known about the assembly, maturation and turnover of these structures.

Initiation of podosome and invadopodia formation

The shift from a quiescent to a migratory phenotype is often characterized by the dissolution of focal adhesions and the formation of podosomes or invadopodia[14,81] at sites of ECM contact (Figure 5). Podosomes and invadopodia have many of the same components as focal adhesions, structures associated with stable attachment to ECM, and whose dissolution is required for cell motility. One study has suggested that there is a reciprocal relationship between focal adhesions and invadopodia, with focal adhesion kinase (FAK) — a scaffold and tyrosine kinase that, together with Src, promotes the turnover of focal adhesions — functioning as a negative regulator of invadopodia through the spatial control of Src[81]. In this view, phosphorylation of FAK at tyrosine 397 promotes its association with Src, and the phosphorylation of Src substrates at focal adhesions. Depletion of FAK releases active Src to promote the phosphorylation of invadopodia proteins and increase invadopodia formation. Other studies have confirmed that inhibition of FAK promotes invadopodia formation[82,83]. However, there are also reports that FAK is present in invadopodia, and that increased FAK expression promotes invadopodia formation[40,84]. Furthermore, the FAK-related kinase Pyk2 is an obligate component of osteoclast podosomes[85–88]. In Src-transformed fibroblasts, the formation of invadopodia is initiated in the vicinity of focal adhesions in response to the focal generation of PI3,4-P_2 ([29]). This lipid recruits Tks5, via association with its PX domain[27]. Mueller and co-workers proposed that aggregation of cortactin at sites of ECM attachment was a key early step in invadopodia formation[15]. Condeelis and co-workers showed that Tks5 co-localized with cortactin to invadopodia precursors, and suggested that Tks5 may be the scaffold that recruits cortactin[89]. In this model, the focal generation of PI3,4-P_2 is the key initiating step in podosome and invadopodia formation, yet we have little idea how this lipid is generated at, or localized to, the membrane near focal adhesions (Figure 5).

How might Tks5 recruit cortactin, and/or orchestrate actin polymerization? Tks5 can bind, directly or indirectly, to the key actin regulators Nck1, Nck2, (N)-WASP and Grb2 ([29,30]). Cortactin also associates with several actin regulatory proteins, including (N)-WASP and Arp2/3, which forms an active complex through cdc42[90], WIP and dynamin, each of which

are required for podosome and invadopodia formation[6]. It seems likely that one or more of these proteins act as a bridge between cortactin and Tks5 and that this is dependent on the phosphorylation status of cortactin. Cortactin is phosphorylated by several kinases, including those of the Src, PAK and ERK families, which regulates its interaction with other proteins[53]. For example, tyrosine phosphorylation of cortactin in response to EGF stimulation causes its dissociation from cofilin, leading to the generation of barbed ends from severed actin filaments, and initiating actin polymerization and invadopodia formation[89] (Figure 5). Subsequent cortactin dephosphorylation blocks the severing ability of cofilin, to enable invadopodia stabilization and degradative capacity[89,90].

Maturation of podosomes and invadopodia

Both cortactin and Tks4 have been shown to regulate secretion and localization of MMPs at invadopodia respectively[10,91,92], and appear to play a role in the maturation of podosomes and invadopodia. In keeping with a key role for cortactin, its recruitment to future sites of ECM degradation immediately precedes the trafficking of proteases to these sites[15]. In contrast, knockdown of Tks5 inhibits invadopodia formation without affecting the secretion of metalloproteases[31]. Loss of the Tks5-related scaffold protein Tks4 has an intermediate phenotype: immature invadopodia (which contain Tks5 and cortactin) are formed and MMPs are secreted, but MT1-MMP fails to localize to the invadopodia, and ECM degradation does not take place[92]. Together, these data suggest a model in which Tks5 and cortactin act to generate invadopodia and secrete metalloproteases, respectively (Figure 5). Tks4 subsequently localizes and/or stabilizes MT1-MMP in the structure to allow focal activation of MMPs and subsequent ECM degradation (Figure 5).

We have already described that the localization of MT1-MMP to podosomes and invadopodia is a key maturation step. A brief description of how MT1-MMP localization is controlled by intracellular trafficking and ECM binding will serve to illustrate the complexity of its regulation. MT1-MMP is delivered to invadopodia in a number of ways. First, a significant fraction derives from endocytic recycling, in a similar manner to integrins. MT1-MMP is efficiently internalized by both clathrin- and caveolae-mediated endocytosis, with trafficking to endosomal and lysosomal compartments for either recycling or degradation. The 20 amino acid cytoplasmic domain of MT1-MMP plays a key role in regulating its endocytosis[93]. Currently attention focuses on Src phosphorylation of MT1-MMP within the AP2-clathrin adaptor binding sequence, which is predicted to impede endocytosis[94]. Src also phosphorylates endophilin A2, reducing its affinity for the GTPase dynamin, thus inhibiting clathrin-mediated endocytosis[94]. Additionally, clustering of MT1-MMP with β1 integrin as a consequence of type-I collagen binding slows endocytosis[95]. Second, MT1-MMP can be mobilized from intracellular stores by a Rab8-dependent secretory pathway[96]. Third, there is an important role for the coordinated actions of the actin cytoskeleton and the exocytic machinery. In this regard, activated Cdc42 and RhoA promote association of the polarity regulator IQGAP1 (which links microtubules with the actin cytoskeleton) and the exocyst complex, which localizes to invadopodia and is required for MT1-MMP localization[97,98]. Since MT1-MMP is not localized at invadopodia in the absence of Tks4 it will be important to determine how Tks4 interfaces with the complex control of MT1-MMP turnover. These data suggest that the MT1-MMP is accumulated at invadopodia through a balance of endocytosis and exocytosis, providing many intervention points to control the cell surface expression of this key protease.

The microtubule network and the actin cytoskeleton are closely linked and cooperate during planar cell migration[99]. Similar cooperation between microtubules and the actin cytoskeleton appears to promote podosome and invadopodia formation and function. For example, microtubules are required for podosome formation and turnover in macrophages and osteoclasts[6] and a recent study showed that the elongation of invadopodia into basement

membranes is dependent on a microtubule network that forms at the base of the structure, suggesting delivery of components to the protruding tip[41] (Figure 5). Furthermore kinesins, motor proteins which transport cargo along microtubules, are required for delivery of MT1-MMP to podosomes[100]. How do the actin and microtubule networks cooperate? Myosin, which helps transport organelles and vesicles along actin, also transports microtubule ends along actin tracks to foci of cortical actin during cell migration[101]. In dendritic cells, myosin inhibition by blebbistatin prevents podosome elongation and invasion[13]. These data suggest that the trafficking of podosome and invadopodia proteins regulates their elongation and invasive capabilities, although it remains to be determined which key proteins are trafficked in this way.

Turnover of podosomes and invadopodia

Podosomes and invadopodia are dynamic structures, with half-lives of actin turnover ranging from minutes to hours. Yet almost nothing is known about how their turnover is regulated. Nor is it clear that the entire structure disassembles. Most studies follow only the turnover of actin, fluorescently-tagged cortactin and AFAP-110 have also been visualized[68,102]. It remains possible that a core structure remaining in the plasma membrane becomes uncoupled from actin polymerization to effect turnover. Alternatively, parts of the structures may turnover rapidly. For example, in invadopodia actin comets the actin head structure turns over rapidly, while the filamentous tail region persists for hours[26]. Protein phosphorylation is important for podosome or invadopodia formation and/or turnover. The tyrosine phosphorylation of Tks5 is critical for invadopodia formation[30], whereas both the tyrosine phosphorylation of cortactin and the serine phosphorylation of AFAP-110 promotes invadopodia turnover[68,89]. Phosphatases likely play key roles in controlling podosome and invadopodia formation and turnover, but few have been characterized to date. PTPε plays a positive role in osteoclasts via dephosphorylation of tyrosine 527 and subsequent activation of Src[103], and PTP1B is required for invadopodia formation[104]. In contrast, the tyrosine phosphatase PTP-PEST negatively regulates invadopodia formation[28]. The 5' inositol phosphatase synaptojanin is localized to invadopodia, and is required for their formation[105], whereas the 3' inositol phosphatase and tumor suppressor PTEN represses invadopodia formation[106]. Finally, the serine protease calpain promotes dendritic cell podosome turnover by cleaving the podosome proteins talin, Pyk2 and WASP[107]. It is important to extend these studies to determine the effect of lipid and protein phosphatases on turnover, as well as isolate other classes of regulators.

PODOSOMES AND INVADOPODIA IN BIOLOGY

Several studies have suggested that podosome proteins might have a role in embryonic development. Furthermore, several human diseases that are a consequence of abnormal cell migration or invasion have been linked to de-regulation of podosome or invadopodia components. These include the genetic diseases Wiskott-Aldrich syndrome and Frank-ter-Haar syndrome, as well as atherosclerosis, tumor progression and metastasis.

Podosomes in development

During development, cells migrate from a centralized location to distal sites in the developing embryo. Cell migration begins in gastrulation and later becomes restricted to certain cell types, particularly those such as neural crest derivatives that have undergone epithelial mesenchymal transitions (EMT). Genes involved in the control of EMT during embryonic development, for example the transcription factors Twist, Snail and Slug, also regulate tumor formation and cancer progression[108–111,112]. Furthermore, invadopodia are frequently found in cancer cells that have undergone EMT, and Twist can promote invadopodia formation[113]. Yet few studies have investigated whether podosomes and/or

podosome-associated proteins are required for embryonic development. Recently, a mutation in the gene encoding the podosome and invadopodia associated protein Tks4 was shown to cause the autosomal recessive disorder Frank-ter-Haar syndrome (FTHS)[114], characterized by skeletal, cardiac, metabolic, and ocular defects, which are replicated in a mouse model of Tks4 loss[114]. These phenotypes are consistent with defects in neural crest-derived cells. To date, it is unclear whether loss of Tks4 results in abnormal podosome formation in FTHS patients or what mechanistic role it plays in this disease. However, we have recently used a zebrafish model to implicate the related protein Tks5 in neural crest cell migration, and detected podosomes in neural crest stem cells in culture (DAM and SAC unpublished). Interestingly, Src family kinases, MT1-MMP, ADAM19, and collagen, all of which are podosome-associated proteins, appear to play a role in cell movement during gastrulation[115–121]. Podosome-associated proteins are also involved in genetic diseases characterized by craniofacial disorders[122]. While it is possible that these defects could be attributed to increased apoptosis or decreased proliferation, it seems more likely that podosome-associated proteins might be required for cell migration during development.

Podosome and invadopodia proteins in disease

Wiskott-Aldrich Syndrome (WAS) is an X-linked recessive disease in which patients present with eczema, thrombocytopenia, and severe immune deficiencies[123]. It is caused by mutations in the *WASP* gene, encoding the actin binding protein WASP, which is predominately expressed in hematopoietic cells[123]. The observation that macrophages and dendritic cells from WAS patients are defective in podosome formation provided the first link between podosomes and a human disease[49]. It has recently been proposed that chemotactic factors cause the recruitment of WASP to focal adhesions, where it acts as a scaffold between integrins and the actin filaments forming in the podosome core[124].

Atherosclerosis is the accumulation of vascular smooth muscle cells in response to injury and vascular stresses such as ischemia, and is attributed to both increased cell proliferation and cell migration. As noted earlier, miRs-143 and -145 control the switch of vascular smooth muscle cells from the differentiated state to the synthetic, motile state[125]. Furthermore, reduced expression of these miRs was noted in aortic aneurysms[77]. Deletion of the gene encoding miR-143 and -145 results in arterial thickening and a blunted response to vasopressive stimuli that is correlated with increased podosome formation and cell migration in primary aortic smooth muscle cells *in vivo* and *in vitro*[19,77]. Interestingly, TGF-β, which is activated by disruption of blood flow and ischemia, induces podosome rosettes in arterial endothelial cells cultured *ex vivo*[20]. Together these data suggest that podosome formation plays a role in vascular homeostasis.

There is a growing body of literature describing the role of invadopodia proteins in invasive cell behavior in tissue culture systems. And several invadopodia proteins are known to be expressed in human cancer tissue. For example, cortactin levels correlate with aggressiveness in squamous cell carcinoma of the head and neck[52]. Tks5 has been detected in several human tumor samples[31]. And sites of invadopodia formation are capable of degrading ECM in human lobular breast carcinoma tissue examined *ex vivo*[126]. But few studies have critically examined the role of invadopodia in tumorigenesis *in vivo*. One of the earliest studies examined AMAP1, an ArfGAP that acts as a bridge between paxillin and cortactin, and co-localizes with them at invadopodia[127]. Expression in breast cancer cells of mutant AMAP1 proteins unable to mediate trimeric complex formation with cortactin and paxillin had only a minor effect on primary tumor growth in mammary fat pads, but a more pronounced effect on subsequent metastasis to the lung. Such a phenotype is consistent with studies demonstrating that invadopodia proteins are typically not required for tumor cell growth *in vitro*. However, an interesting complexity has emerged from the *in vivo* study of other invadopodia proteins. For example, inhibition of MMPs prevents primary tumor

growth in a variety of mouse models[128]. Over-expression of MT1-MMP in cancer cells promotes tumorigenesis, and conversely, inhibition of the enzyme reduces tumor growth as well as invasion[129]. Knockdown of cortactin impairs tumor growth in a model of head-and-neck cancer[32], and knockdown of Tks5 also impairs primary tumor growth[51].

Why the discrepancy between the cell based and animal models? Consideration of data derived from 3-dimensional (3D) culture systems perhaps offers a way to rationalize the data. Tumor cell growth in 3D matrices of type I collagen requires MT1-MMP and metalloprotease activity, whereas growth in 2D on top of type I collagen does not[48,129]. Likewise, cortactin promotes growth in the 3D culture environments of agarose and matrigel[52]. The serine/threonine kinase LIMK, and the adaptors Tks4 and Tks5 are also required for efficient growth in, but not on top of, 3D collagen ([130] and Barbara Blouw, Matthew Buschman, Begona Diaz and SAC, unpublished). The mechanism(s) by which invadopodia are required for 3D growth has not yet been fully explored, but might involve the induction of proteolysis. In the case of cortactin knockdown, growth can be rescued by co-culture with cortactin-expressing cells, suggesting that invadopodia-directed proteolysis may release autocrine growth factors from the cell surface[52]. Focal ECM degradation might also promote ECM–integrin signaling, and be necessary for expansion of the tumor into the microenvironment. *In vivo*, invadopodia-mediated regulation of pericellular proteolysis could also be responsible for the regulated production of pro-angiogenic factors such as VEGF, which might explain the reduced angiogenesis observed in Tks5 knockdown tumors[131]. Finally, many of the stromal cells present in the tumor microenvironment, including macrophages, fibroblasts, and endothelial cells, can form podosomes. It will be interesting to determine what role podosomes might play in tumor progression.

Conclusions/Perspectives

Tremendous strides have been made in our understanding of podosomes and invadopodia in recent years, yet many important questions remain. Are there molecular differences between the two structures? What role do they play in the mechanics of cell migration? How many cell types elaborate podosomes during development? What are the master switches that control their formation and function? What role does the ECM play in formation of these structures? How is pericellular proteolysis controlled by podosomes and invadopodia? Which diseases might be caused by alterations in podosome biology? How do invadopodia promote tumor growth and progression?

Even as we address these questions, there is already strong circumstantial evidence to suggest that modulation of podosomes and invadopodia might represent a viable therapeutic strategy to alter the course of several diseases. For example, podosome and invadopodia inhibitors might have value in the treatment of atherosclerosis and cancer. Transient activators of podosome formation might also ameliorate the progressive skeletal anomalies of FTHS infants and perhaps other craniofacial syndromes, as well as immunodeficiency. Many of the known components of podosomes and invadopodia are scaffolding or adaptor proteins with no catalytic activity, and are therefore unlikely drug targets. In the future, it will be important to identify key enzymatic regulators, with an eye to the generation of novel targeted therapies.

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GLOSSARY

Epithelial mesenchymal transitions;

PX domain	a protein domain of approximately 120 amino acids that associates with phosphatidylinositol lipids
barbed end	the end of an F-actin polymer to which a G-actin monomer is attached
lamellipodia	actin based projections of the leading edge of a migrating cell
actin cloud	a concentration of actin that surrounds podosome cores in osteoclasts
podosome belt	a fusion of podosomes at the periphery of osteoclasts cultured in 2D
outside-in signalling	the transmission of extracellular signals to the cytoplasm by the engagement of integrin receptors with the ECM
inside-out signaling	the transmission of the status of the cell to the extracellular space via integrin receptors

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Box 1. Highlights from 25 years of Podosome/Invadopodia Research

1985:	Actin- and phosphotyrosine-rich ventral protrusions are recognized as cell attachment points to the ECM, and called podosomes[2].
1985:	Src is localized to the sites of cell contact to ECM, and it is shown that ECM degradation occurs at these sites[3].
1988:	Podosomes are found in osteoclasts adhering to bone laminae[5]
1989:	The Src-enriched sites of degradation are shown to be identical to the actin-rich protrusions known as podosomes – the new name invadopodia is coined[4].
1990:	Bone resorption by osteoclasts is shown to require the podosome belt - the first physiologic process shown to require podosomes[132].
1994:	First description of invadopodia-dependent proteolytic activity in human cancer cells[133].
1997:	MT1-MMP is located in podosomes and invadopodia and is required for cancer cell invasion[134,135].
1999:	First demonstration of a podosome-associated disease - Wiskott-Aldrich syndrome[49].
2000:	Microtubules are required for podosome formation[136].
2005:	The adaptor protein Tks5 is shown to promote invadopodia formation, and be required for invasive behavior of human cancer cells[31].
2006–2008:	In vivo studies demonstrate that invadopodia-associated proteins are required for both tumor growth and metastasis[51,53].
2008:	ECM rigidity promotes invadopodia activity[40].
2009:	Reactive oxygen species are necessary for podosome and invadopodia formation[28].
2009–2010:	First demonstration that vascular pathophysiology involves podosome formation[19,20], and that podosomes exist in vivo[19].
2010:	Detailed visualization of invadopodia elongation as cells traverse basement membrane[41].
2010:	First description of podosome structure and invasion in 3D, demonstrating that podosomes invade into the ECM using a mechanism similar to invadopodia[13].

SUMMARY

- Podosomes and invadopodia are actin-based dynamic protrusions of the plasma membrane. They act as sites of attachment to, and degradation of, the extracellular matrix.
- These structures contain actin regulators such as cortactin and (N)-WASP, adaptor proteins such as Tks4 and Tks5, and several pericellular proteases.
- Podosomes are found in vascular smooth muscle and endothelial cells, as well as cells derived from monocyte lineages. Their presence correlates with migratory ability.
- Invadopodia are found in invasive human cancer cells. In 2D culture, their presence correlates with invasive behavior. However, in 3D culture and in vivo, invadopodia proteins are required for cell growth.
- Podosome proteins have been implicated in human developmental and immune disorders and dysregulation of podosome formation is associated with atherosclerosis.
- Small molecule regulation of podosomes and invadopodia might represent a novel therapeutic strategy to treat several diseases.

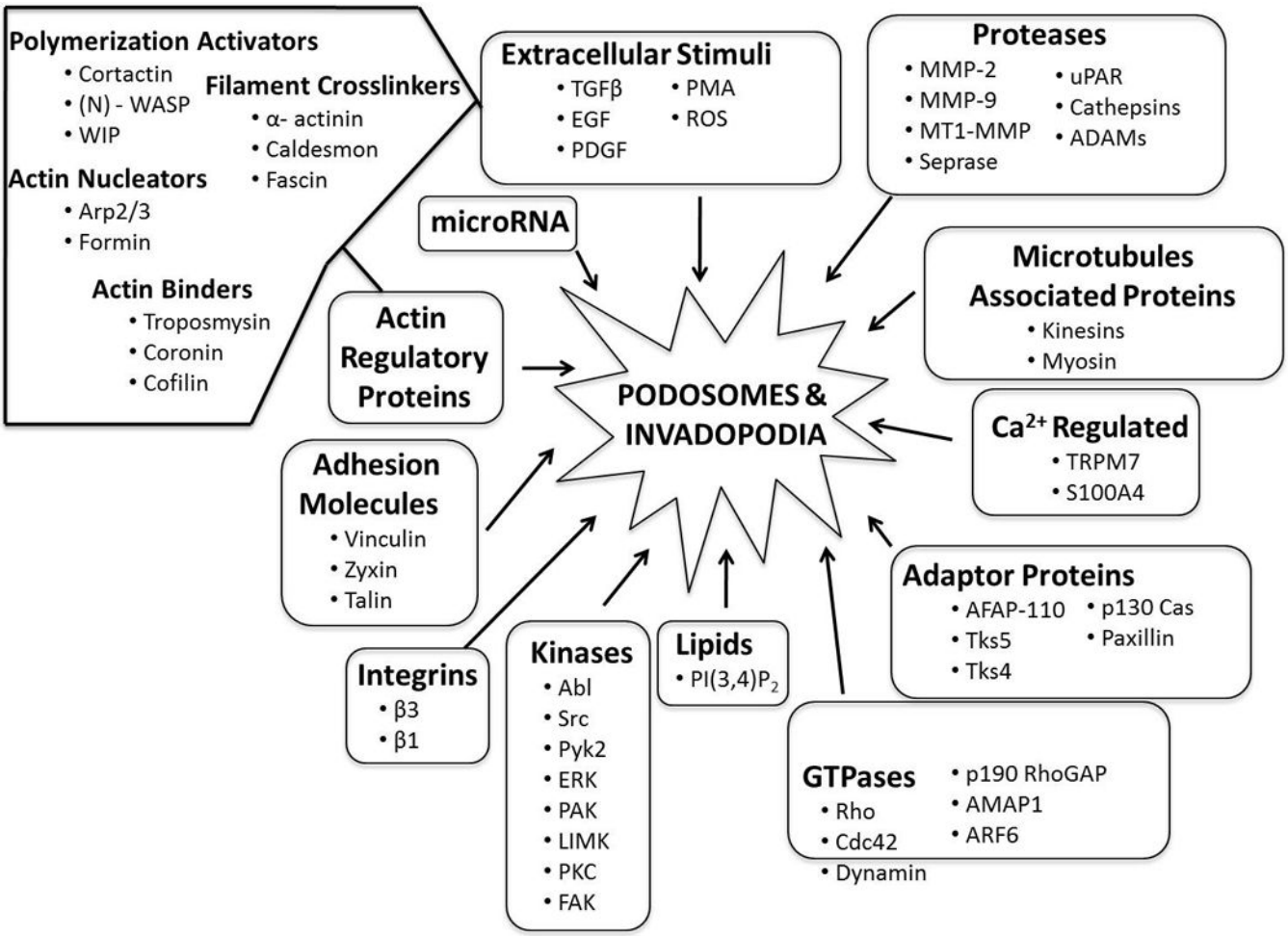

Figure 1. Regulators of Podosome and Invadopodia Formation

There are several components within the cell that are regulated to induce the formation and promote the function of podosomes and invadopodia. Some of the main components that are required for these structures are highlighted are, however this is not intended to be a totally comprehensive list.

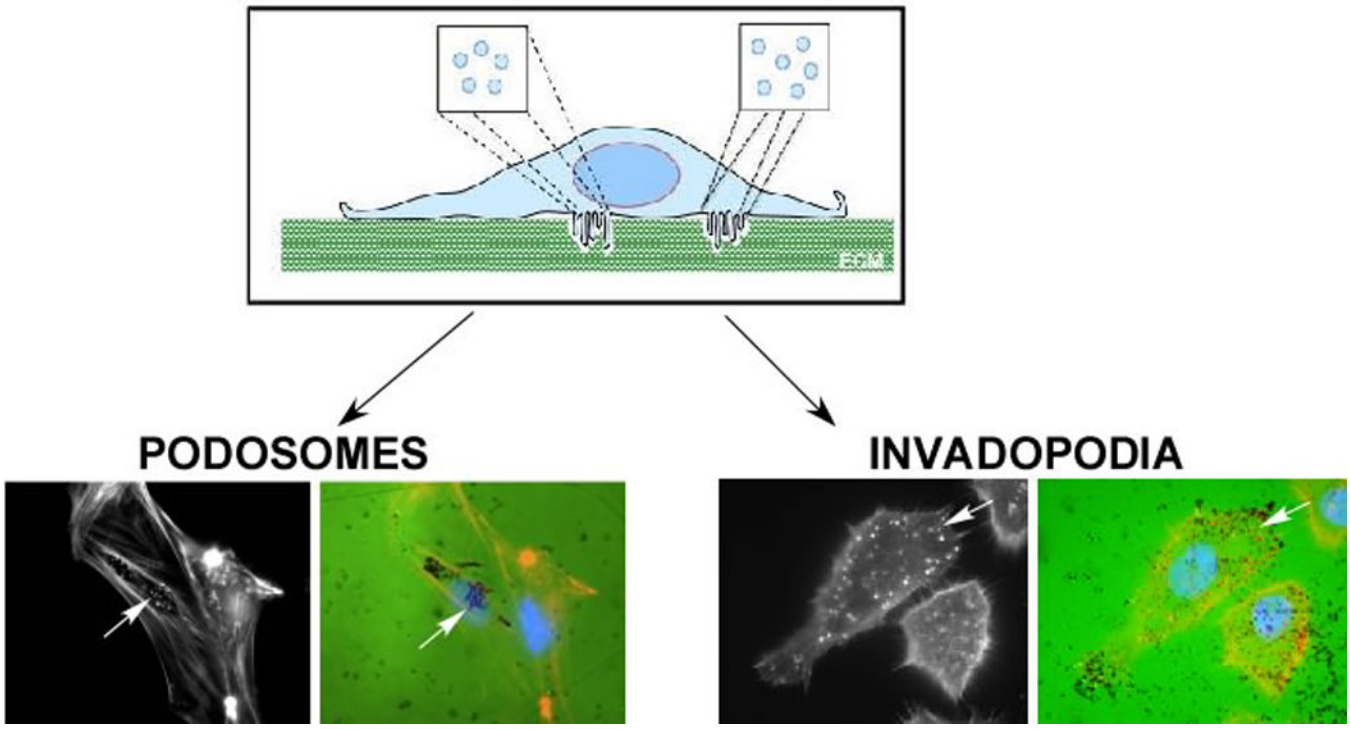

Figure 2. Structure and Function of Podosomes and Invadopodia

Podosomes and invadopodia are actin rich structures that are formed on the ventral membrane of the cell. These structures are often seen as individual puncta or rosettes that protrude into the extracellular matrix (ECM). Classically, presence of these structures is often confirmed by culturing cells on top of fluorescently-conjugated matrix (FITC-gelatin), staining cells for F-actin and examining co-localization between F-actin puncta and degradation of matrix (black regions). This is demonstrated in vascular smooth muscle cells (podosomes) and SCC61 head and neck squamous carcinoma cells (invadopodia) as indicated by arrows.

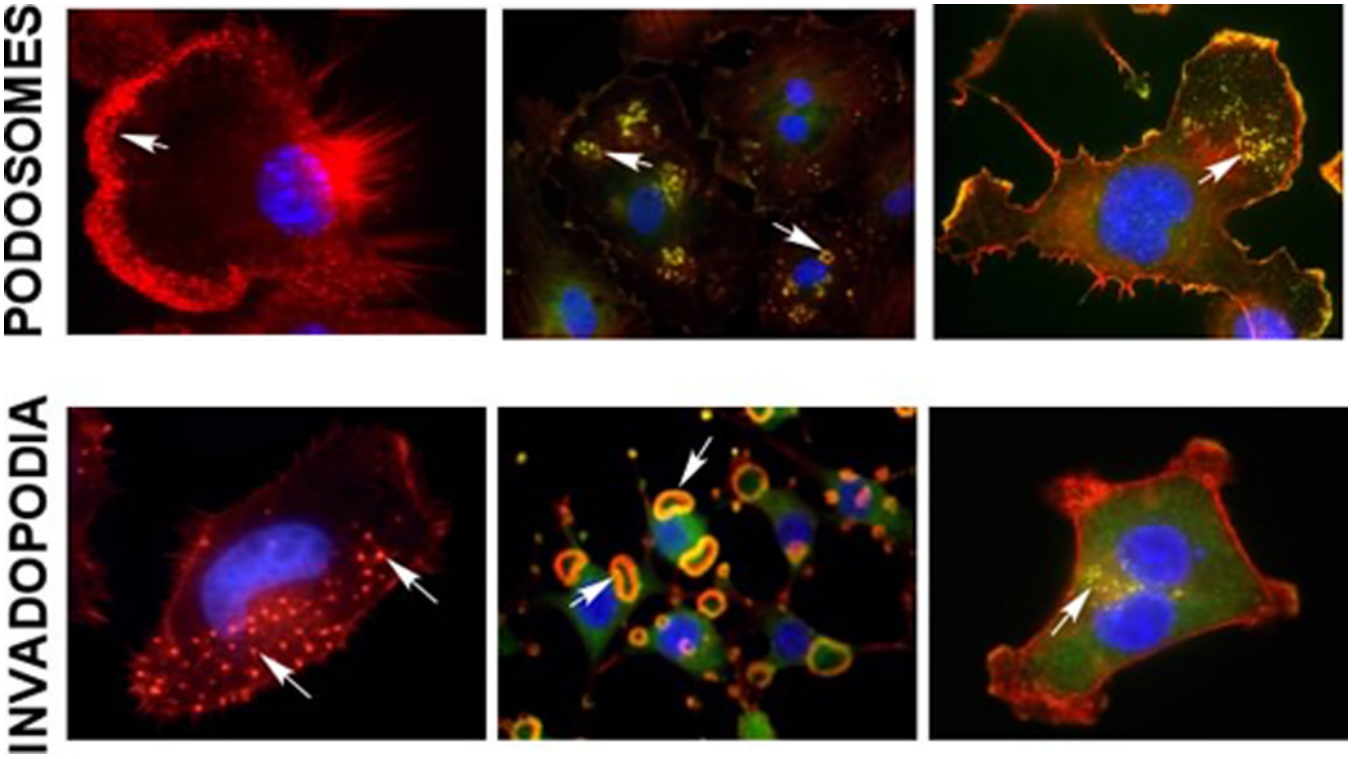

Figure 3. Podosomes and Invadopodia in 2-dimensions

Formation of podosomes and invadopodia is frequently visualized by co-staining cells with F-actin (red) and the podosome and invadopodia associated protein cortactin (green). These structures can be seen in many cell types. Podosomes (top row): Macrophages (IC-21), vascular smooth muscle cells (VSMCs - A7r5 treated with 25nM PDGF) and neural crest stem cells (JOMA1.3, treated with 20nM PMA). Invadopodia (bottom row): head and neck squamous carcinoma cells (SCC61), Src-3T3 cells, and breast cancer cells (MDA-MB-231). Arrows denote podosomes and invadopodia.

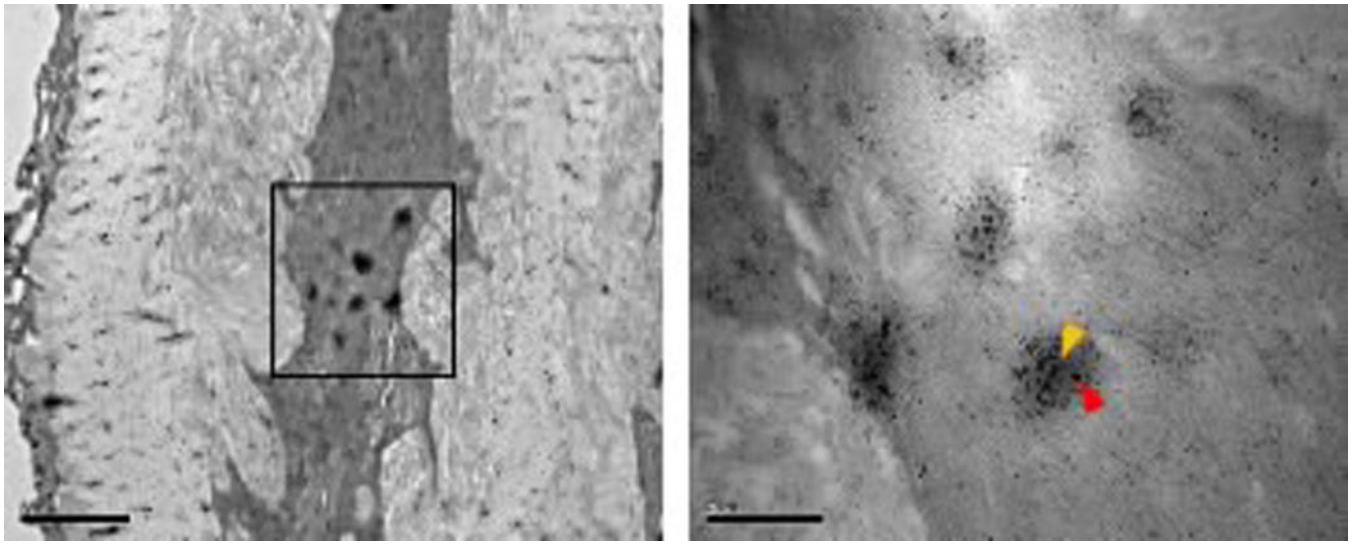

Figure 4. Podosomes in vivo

Podosome structures have recently been observed in vivo. (A) Immunoelectron microscopy for Tks5 (red arrowhead) and cortactin (yellow arrowhead) in murine VSMCs in vivo. Enlarged image of labeled aorta section (boxed area, right panel) (B) Protrusive structures visible in migratory trunk neural crest cells in (*foxd3*:GFP) zebrafish, *Danio rerio*.

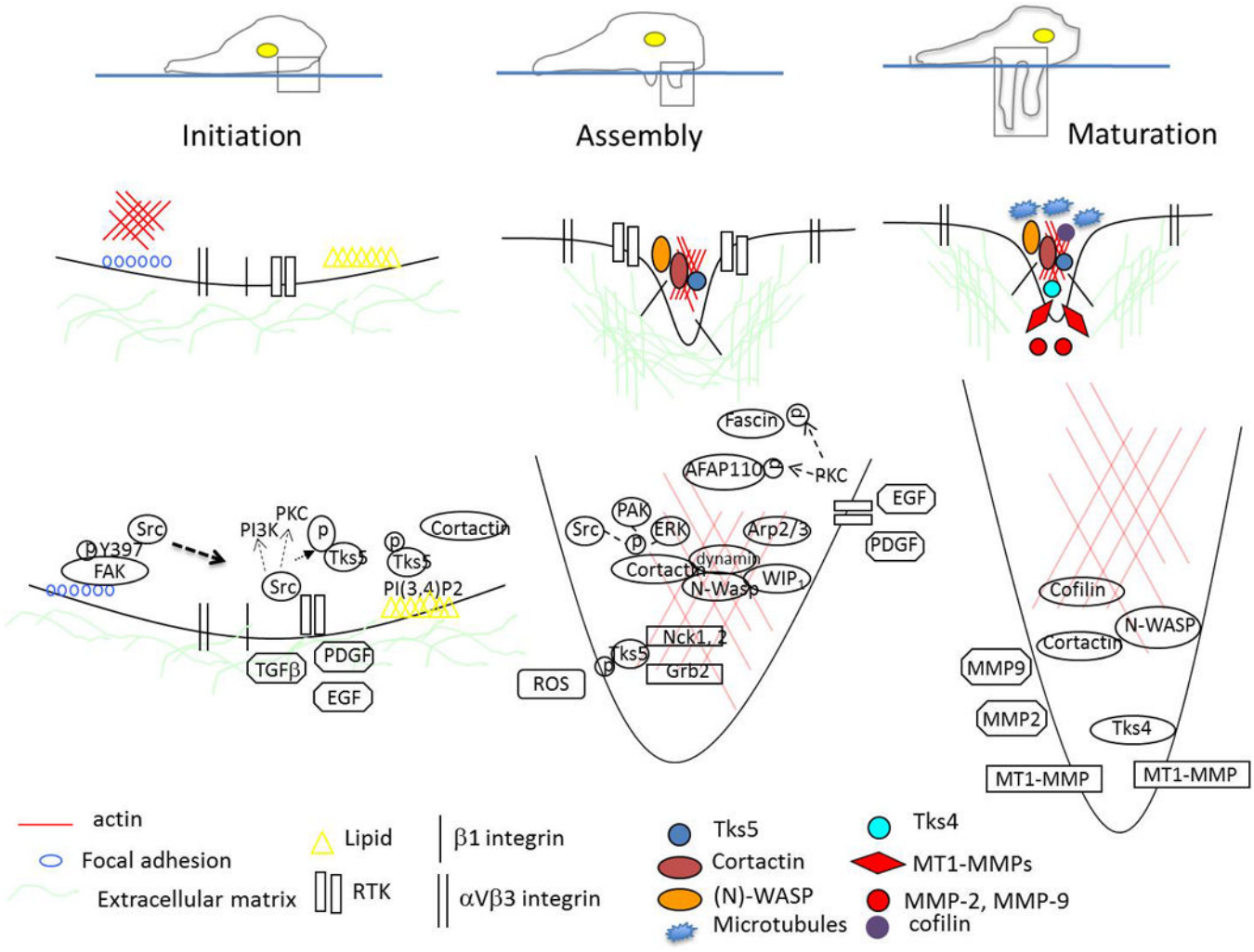

Figure 5. The stages of invadopodia formation

Initiation - Cells establish focal adhesions with the ECM through interaction of integrins, Src, and FAK (left side). In migrating cells, a switch occurs that will release Src to bind Tks5 and localize to regions containing $PI(3,4)P_2$. These intracellular changes are initiated by factors such as EGF, PDGF, TGFβ (right side).

Assembly - Formation of invadopodia occurs through recruitment and activation of actin regulatory proteins (Arp2/3, WIP), phosphorylation of key invadopodia components (cortactin, Tks5, fascin, AFAP110), and production of ROS.

Maturation - Invadopodia promote degradation of ECM by coordinating secretion of MMP-2 and MMP-9, and enabling delivery (potentially through microtubules) and presentation of MT1-MMP to the tip of the protruding structure through the interaction of key invadopodia components (cortactin, Tks4, β1 integrin).

	Description	Location	Protrusive Dimension	Actin Rearrangement	Lipids Involved	Microtubule Dependence	Pericellular Proteolysis	Duration of Structure
Lamellipodia[137,138]	Surface-attached, sheet-like protrusions	Leading edge of the cell	Width: 0.1 – 0.2 μm	Branched actin filaments	Yes PI(4,5)P2[139]	Induces lamellipodia extension[140]	Minimal[141]	Minutes[142]
Filopodia[137,138]	Finger-like projections	Usually located in regions of lamellipodia	Width: 0.1 – 0.3 μm Length: 3 –10 μm	Parallel actin filament bundles	Yes PI(4,5)P2, PI(3,4,5)P3	Regulates filopodia density in lamellipodia rich areas[143]	No	Minutes
Focal Adhesions[144,145]	Large clusters of transmembrane receptors, integrins, and cytosolic proteins that connect ECM and actin cytoskeleton	Leading edge of the cell	Width: 2 – 6 μm	Predominately parallel actin filament bundles but branched actin at end[146]	Yes PI(4,5)P2	Inhibition of microtubules leads to increased focal adhesions[147]	Minimal	Hours – depending on rate of cell migration
Podosomes[6]	Actin rich core that is surrounded by a ring of actin- associated and signaling proteins. Term applies to normal cells	Ventral cell surface Often clustered behind the leading edge of the cell	Width: 0.5 – 2 μm Length: > 2 μm	Branched and unbranched actin[39]	Yes PI(3,4,5)P3[148]	Required for elongation and/or formation [13,136]	Yes MT1-MMP, uPAR[13,149]	Minutes
Invadopodia[6,11]	Actin rich core that is surrounded by a ring of actin- associated and signaling proteins. Term applies to normal cells	Ventral cell surface Often situated under the nucleus	Width: 0.5 – 2 μm Length: > 2 μm	Branched actin at the cell surface. Unbranched actin through the tip of the protrusion[41]	Yes PI(3,4)P2, PI(3)P, lipid rafts (Cav-1)[29,92]	Required for elongation but not formation[41]	Yes MMP2, MMP9, MT1-MMP, seprase, uPAR ADAMs 12, 15, 19,	Hours