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COMMENTARY

The microenvironment controls invadosome plasticity

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ABSTRACT

Invadosomes are actin-based structures involved in extracellular matrix degradation. Invadosomes is a term that includes podosomes and invadopodia, which decorate normal and tumour cells, respectively. They are mainly organised into dots or rosettes, and podosomes and invadopodia are often compared and contrasted. Various internal or external stimuli have been shown to induce their formation and/or activity. In this Commentary, we address the impact of the microenvironment and the role of matrix receptors on the formation, and dynamic and degradative activities of invadosomes. In particular, we highlight recent findings regarding the role of type I collagen fibrils in inducing the formation of a new linear organisation of invadosomes. We will also discuss invadosome plasticity more generally and emphasise its physio-pathological relevance.

KEY WORDS: Invadopodia, Invadosome, Cell invasion, Collagen, Matrix degradation, Podosome

Introduction

Remodelling and invasion of the extracellular matrix (ECM) by cells are crucial in physiological and pathological conditions. Under physiological conditions, ECM remodelling is necessary during foetus implantation, embryogenesis, bone homeostasis and wound repair. Moreover, cell invasion associated with the crossing of anatomic barriers such as endothelial basement membrane is a basic function of immune cells to respond to and prevent infection. However, tumour cell invasion is instrumental in the formation of metastases, the major cause of cancer-related death.

Invadosomes, including podosomes and invadopodia, are F-actin-based structures that are capable to interact with and to degrade the ECM. Although widely studied, the features that distinguish invadopodia from podosomes are vague, but have stimulated intense debates in the field. In order to accurately define their respective intrinsic characteristics, parameters, such as the cellular model, the microenvironment matrix as well as the cytokines it contains, and thus the internal or external stimuli necessary for their induction, need to be characterised.

The number of reports describing invadosomes, in a plethora of situations, is increasing. Similarities between the different structures of invadosomes have been described. Indeed, all invadosome structures contain actin, matrix receptors and proteases. Many studies have described the molecular composition of invadosomes using different approaches (Attanasio et al., 2011; Cervero et al., 2012). However, similar to focal adhesions, invadosomes connect cells with the ECM through contact foci consisting of large multiprotein complexes. Thus, owing to the dynamic nature of this complex, identifying the full molecular identity of these structures is challenging (Artym et al., 2015; Beaty et al., 2013; Sharma et al., 2013; Valenzuela-Iglesias et al., 2015). Currently, there is no study that is able to define the invadosome proteome as accurately as has been done for focal adhesions (Goicoechea et al., 2014; Robertson et al., 2015). In addition, there are only few studies describing the very existence and role of invadopodia *in vivo*. The main objectives of this Commentary are to define the different structures that have been described as invadosomes, to illustrate invadosome plasticity as well as their physio-pathological relevance and to identify the future avenues that need to be explored in order to fully understand the complexity of the invadosome biology.

General features of invadosomes

Normal and cancer cells interact physically with their microenvironment through anchoring or adhesive molecular structures that respond to different stimuli, such as mechanical or chemical cues, by remodelling their shape and their ECM adhesion capacity. Owing to the diversity of the ECM, cells need to constantly adapt their adhesion capacity to the matrix microenvironment. To attach to the ECM, cells form different adhesion structures, such as focal adhesions or invadosomes. In this Commentary, we will focus only on invadosomes, but it is worth noting that focal adhesions have been recently described to also promote matrix proteolysis in fibrosarcoma cells (Wang and McNiven, 2012).

Invadosomes are microdomains that are formed at the ventral surface of the cell (Artym et al., 2006; Guegan et al., 2008). The basic unit of an invadosome corresponds to an F-actin core surrounded by a ring of regulatory and adhesive molecules. The F-actin-core is enriched in actin-regulating proteins, including cortactin, neural Wiskott–Aldrich syndrome protein (N-WASP, also known as WASL) and Arp2/3. The ring is composed of actin-associated proteins that are also found in focal adhesions, such as integrins, talin, vinculin and paxillin (Linder et al., 2011). Invadosomes can be observed in different conformations, such as aggregates (Fig. 1A), individual dots (Fig. 1B), rosettes (Fig. 1C) or linear structures (Fig. 1D).

In invadosome aggregates, the rings link the invadosome cores (Fig. 1A). The ring contains a combination of two subsets of unbranched actin filaments that contain myosin and appear to be the basis for actomyosin contractility, either at individual invadosomes (i.e. the lateral fibres) or between invadosome cores (the connecting cables) (Linder and Wiesner, 2015; Luxenburg et al., 2007; van den Dries et al., 2013) (Fig. 1A). In invadosomes that form individual dots, the ring is present in normal cells such as endothelial cells (Moreau et al., 2003), but not easily observed in cancer cells (Fig. 1B). Rosette arrangement has been observed in various cell

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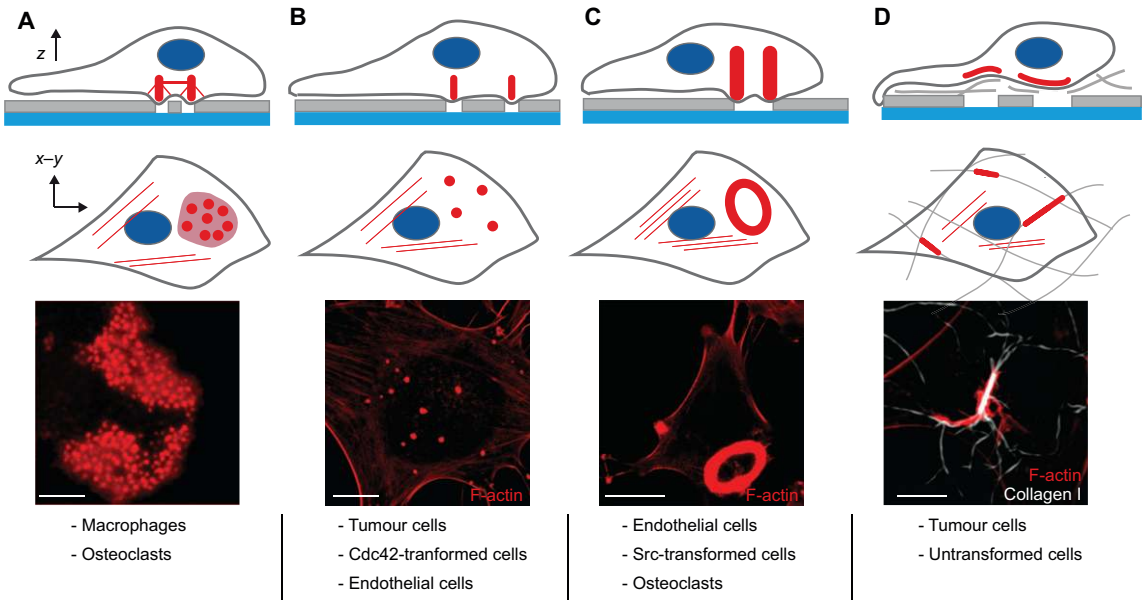

Fig. 1. Different organisations of invadosomes. Schematic representation of the different invadosome organisations in *z* and *x*–*y* directions (top), together with a representative confocal microscopy image (bottom). (A) Invadosomes organised in aggregates. (B) Invadosomes organised in dots. (C) Invadosomes organised in a rosette. (D) Linear invadosomes formed along type I collagen fibrils. F-actin is shown in red, and type I collagen fibrils in grey (D). Scale bars: 10 µm.

models, such as osteoclasts, endothelial cells or Src-transformed fibroblasts (Destaing et al., 2003; Seals et al., 2005; Varon et al., 2006a). These rosettes can be considered as a condensation and reorganisation of the basic invadosome units (Fig. 1C). A linear organisation for invadosomes has been observed in cells that were seeded onto type I collagen fibrils (Fig. 1D) (Juin et al., 2012). Invadosome size, their number per cell and their half-life are highly variable and depend on the cell type and its microenvironment (Artym et al., 2011; Destaing et al., 2003; Gimona et al., 2008; Linder, 2007; Schoumacher et al., 2010).

Invadosomes have the dual capacity to interact with and degrade the ECM using matrix metalloproteinases (MMPs), such as MT1-MMP (also known as MMP14), MMP2 and/or MMP9 (Linder, 2007). Invadosomes in cancer cells have been shown to be more efficient in degrading the matrix than in untransformed cells (Linder, 2007; Murphy and Courtneidge, 2011). Although this is clearly of interest, it is, however, difficult to quantify and compare the matrix-degradation capacity of cancer cells with that of other invadosome-containing cells, such as osteoclasts or macrophages. In fact, osteoclasts have an impressive ability to degrade bone, and macrophages can easily degrade ECM in order to cross anatomical barriers.

As previously described for focal adhesions (Shemesh et al., 2005), invadosomes also act as matrix mechanosensors (Destaing et al., 2011). Indeed, invadosomes can sense and respond to a modulation of ECM stiffness (Alexander et al., 2008; Collin et al., 2008, 2006; Destaing et al., 2011; Juin et al., 2013; van den Dries et al., 2014). Moreover, using an innovative microscopy technique, Labernadie et al. have demonstrated that human macrophage invadosomes are able to generate a protrusion force that increases with the stiffness of the ECM (Labernadie et al., 2014, 2010).

Induction of invadosome formation

In addition to their occurrence in both normal and cancer cells, invadosomes can be separated in two classes based on whether they are constitutively present in cells or are inducible. In myelomonocytic cell types, such as macrophages, dendritic cells, neutrophils and osteoclasts, invadosomes arise spontaneously upon cell adhesion (Linder et al., 2000; Saltel et al., 2006). However, in these cell types, differentiation and adhesion stimuli are prerequisites for the formation of invadosomes. For example, monocytes are unable to form invadosomes spontaneously and have to be stimulated with macrophage-colony-stimulating factor (M-CSF) to induce their formation.

Some non-hematopoietic cells, including endothelial cells, can also form invadosomes upon appropriate stimulation, such as expression of a constitutively active form of Cdc42 (Moreau et al., 2003), upon c-Src activation or following treatment with *phorbol esters* or sodium fluoride (Goicoechea et al., 2014; Kaverina et al., 2003; Tatin et al., 2010, 2006), as well as after treatment with various cytokines, such as transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF) and tumour necrosis factor α (TNFα, also known as TNF) (Osiak et al., 2005; Varon et al., 2006b). Similarly, cancer cells can exhibit either constitutive or inducible invadosomes (Saltel et al., 2011). Indeed, not all cancer cell lines show constitutive invadosomes, and invadosome formation can be stimulated by various stimuli, such as treatment with TGF-β or epidermal growth factor (EGF) (Mader et al., 2011; Mandal et al., 2008).

Recently, we have shown that type I collagen fibrils are a potent inducer of a new type of invadosomes, which we termed linear invadosomes (Fig. 1D). These linear invadosomes are formed along type I collagen fibrils and are found in both normal and transformed cells (Di Martino et al., 2015; Juin et al., 2012; Juin et al., 2014; Schachtner et al., 2013). Linear invadosomes present markers that are common to all invadosomes, such as the actin-binding proteins N-WASP and cortactin (Artym et al., 2006), the scaffold protein tyrosine kinase substrate 5 (Tks5, also known as SH3PXD2A) (Blouw et al., 2015) and the Rho GTPase Cdc42 (Di Martino et al., 2014). Linear invadosomes, in contrast to invadosomes that are

organised into individual dots, do not contain ring proteins, such as talin, vinculin, paxillin or integrins (Juin et al., 2012). We have demonstrated that linear invadosomes that are induced by type I collagen fibrils depend on discoidin domain receptor 1 (DDR1). In addition to representing a new organisation of invadosomes, linear invadosomes provide evidence for a structural link between invadosomes because they are seen both in normal and cancer cells. All invadosomes, regardless of whether they occur in aggregates, dots, rosettes or the linear conformation, have the same ECM-degrading function and common markers, such as F-actin, Tks5, MMPs and the RhoGTPase Cdc42, suggesting that they are all variations of the same functional entity. Therefore, the main differences observed between different invadosomes might be due to the varying cell models in which they are found, but, above all, could arise from the way in which invadosome structures are induced in these cells. Regardless of these variations, adhesion to the ECM, which is mediated by ECM receptors, is a prerequisite for invadosome formation. In the next section, we discuss the various ECM receptors that have been implicated in invadosome formation.

Effects of receptors and ECM on invadosomes

The repertoire of cell surface receptors involved in invadosome formation varies and is modulated depending on the nature of the ECM components that are encountered by the cell; these include laminin and type IV collagen of the basement membrane, or fibronectin, tenascin, hyaluronic acid and type I collagen of the interstitial ECM. Type I collagen is the major element of connective tissue and is highly abundant, particularly in tissues, such as bone, dermis or tendon. Moreover, type I collagen is overexpressed (Ramaswamy et al., 2003) and often drastically remodelled in cancer (Conklin et al., 2011; Levental et al., 2009). However, not much is known about the receptors that are present at invadosome structures, mainly owing to the fact that most of the invadosome studies have been carried out in non-physiological matrices. In contrast to focal adhesions, in which integrins are the major ECM receptors, a number of receptors, including integrins, CD44 and discoidin domain receptors (DDRs), have been shown to bind to the matrix at invadosomes. This variability reflects the ability of invadosome to form in a variety of cells that are exposed to different matrices.

Integrins

Integrins are the most studied ECM receptors. This receptor family is clustered into 18 α-subunits and eight β-subunits that are able to form 24 heterodimers that interact with specific matrix components, such as collagens, laminins, fibronectin and vitronectin (Barczyk et al., 2010; Humphries et al., 2006). Integrins are usually observed in invadosomes in normal cells. For example, the integrin αvβ3 had been detected in the ring surrounding the invadosome cores in osteoclasts (Pfaff and Jurdic, 2001; Zambonin-Zallone et al., 1989). Moreover, ablation of β1, β2 and β3 integrins was necessary to inhibit invadosome formation in osteoclasts (Schmidt et al., 2011). In addition, a crucial role for β2 integrins in invadosome formation and dynamics has been demonstrated in dendritic cells, smooth muscle cells and macrophages (Duong and Rodan, 2000; Gawden-Bone et al., 2014; Kaverina et al., 2003). β1 integrin is necessary for invadosome formation in Src-transformed fibroblasts and in endothelial cells (Destaing et al., 2010; Poincloux et al., 2011; Seano et al., 2014). However, the situation is less clear in cancer cells where the integrin-containing ring is not systematically detectable. This could depend on the specific model, the invadosome maturation steps and the matrix context (Beaty and Condeelis, 2014; Branch et al., 2012). Here, other adhesion molecules that interact with integrins, such as vinculin or paxillin, can be used to visualise the invadosome ring (Pfaff and Jurdic, 2001). Furthermore, depending on the cancer cell type and the matrix context, these adhesion factors can be found either at the invadosome ring (Chan et al., 2009; Mueller et al., 1999), or within the invadosome core (Beaty et al., 2013). Most studies of cancer cells point to β1 integrins as the relevant ECM receptor. However, owing to the high number of possible integrin combinations, other integrins could also be important for invadosome formation, depending on the ECM context and the integrin repertoire of the specific cell. For example the laminin-interacting β4 integrin is found at the basis of the actin core in invadosome-like structures in epithelial cells (Spinardi et al., 2004).

CD44

The hyaluronan receptor CD44 is found in a large number of cells, such as fibroblasts, epithelial cells and endothelial cells. It is also used as a cancer stem cell marker and is involved in cancer cell invasion (Hiraga et al., 2013; Jaggupilli and Elkord, 2012). CD44 not only interacts with hyaluronic acid, but also with collagen, osteopontin and MMPs. CD44, in addition to integrins, has been observed in invadosomes and shown to be involved in their formation. CD44 has been found at the basal part of invadosome cores in osteoclasts and macrophages (Chabadel et al., 2007; Chellaiah and Ma, 2013; Van Goethem et al., 2011), whereas integrins are found at the ventral part of the invadosome ring in these cells. The spatial separation of CD44 and integrins suggests that they could have different roles within the same invadosome structure, with integrins ensuring its adhesion function and CD44 being involved in degradation activity (Chabadel et al., 2007). In other examples, such as in some cancer cells, CD44 localises to the basal part of invadosome core and is important for their formation (Grass et al., 2013; Lagarrigue et al., 2010; Vikesaa et al., 2006).

DDRs

DDRs are a ubiquitously expressed family of receptors known to interact with fibrillar collagens, in particular type I collagen. The DDR family is composed of two members, DDR1 and DDR2. Unlike integrins and CD44, DDRs are receptor tyrosine kinases. DDRs are considered to be collagen sensors and are involved in different cellular processes, such as cell differentiation, adhesion, migration and invasion (Leitinger, 2014). DDR1 and DDR2 are also deregulated in various cancers, such as breast and lung cancers (Valiathan et al., 2012). Independently of its collagen-binding activity, DDR1 has been shown to suppress actomyosin contractility at cell–cell contacts during collective cell migration (Hidalgo-Carcedo et al., 2011). DDR2 activation by collagen regulates the stability of SNAIL1 (also known as SNAI1) by stimulating ERK2 (also known as MAPK1) activity and so facilitates breast cancer metastasis (Zhang et al., 2013). As mentioned above, we have discovered that type I collagen fibrils are powerful and physiological inducers of linear invadosomes in various cell types, including cancer cells that have no constitutive invadosomes (Juin et al., 2012). Interestingly, neither β1 integrin nor CD44 are necessary for linear invadosome formation (Juin et al., 2012), which depends on collagen-dependent activation of DDR1 (Juin et al., 2014) (Fig. 2).

It should be noted, however, that it is possible that other receptor families, such as the syndecans, might also be involved in invadosome formation (Aga et al., 2008). Several studies have

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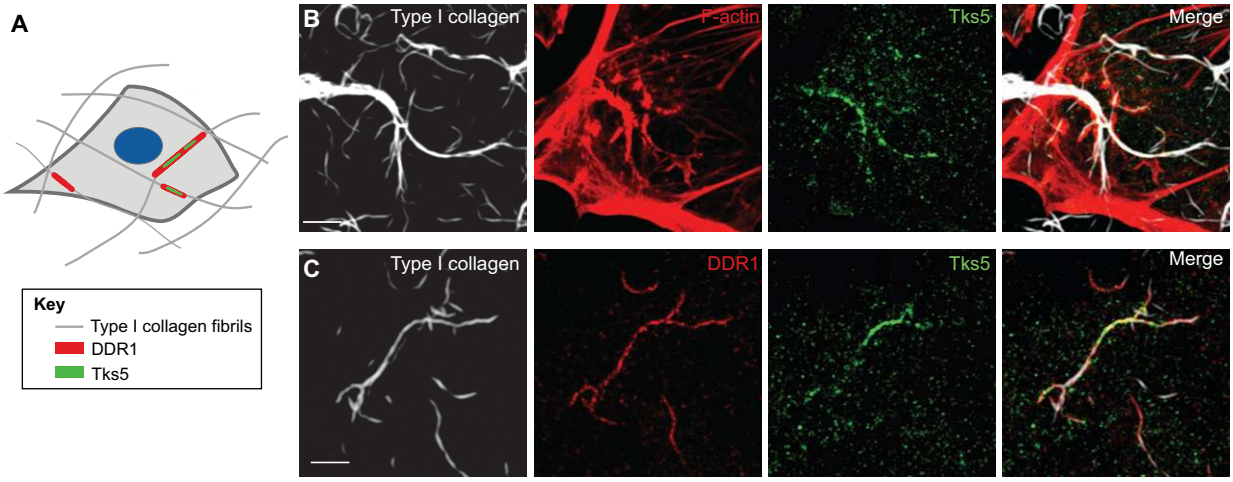

Fig. 2. Composition and organisation of linear invadosomes. (A) Schematic representation of a cell seeded into type I collagen fibrils. (B) Confocal microscopy images of MDA-MB-231 cells illustrate the molecular organisation of linear invadosomes, F-actin (in red) and Tks5 (in green) colocalise along type I collagen fibrils (in grey). (C) Linear invadosomes with different stainings, DDR1 (in red) and Tks5 (in green) colocalise along the collagen fibrils (in grey). Scale bars: 5 µm.

demonstrated that there is crosstalk between integrins and CD44, and between integrins and DDR1 (Fujisaki et al., 1999; Xu et al., 2012). In fact, a combination of these different receptor types might be necessary to regulate the formation and the functions of invadosomes.

As integrins are clearly not the only means to form invadosomes, it is important to decipher the invadosome–adhesome in the context of a complex and physiological matrix.

Invadosome plasticity

As some of the invadosome components are common to the different types of invadosomes that have been described, the existence of a common precursor has been suggested (Boateng and Huttenlocher, 2012; Gimona et al., 2008; Linder, 2009; Linder et al., 2011). Here, we wish to raise the possibility that, in reality, this apparent variability in fact represents the ability of a common invadosome precursor to adapt to its microenvironment, for example to modulate its morphology according to the substrate it encounters, such as taking on a linear shape along a type I collagen fibril. It is worth noting that invadosome structures were initially classified based solely on their appearance and independently of the respective ECM substrate present or regardless of which stimuli were involved in their formation (such as active Cdc42, TGF-β stimulation, Src activation, etc.). Indeed, type I collagen can induce either *de novo* formation or a reorganisation of pre-existing invadosomes (Juin et al., 2012). Therefore, depending on the means of stimulation, the same cell is able to organise its actin cytoskeleton into different forms of invadosomes, such as either classical dot-like or linear invadosomes (Fig. 3). Indeed, NIH-3T3 fibroblasts that do not present classical invadosomes under basal conditions (Fig. 3B) form (1) dots when Cdc42 is activated (Di Martino et al., 2014) (Fig. 3C), (2) rosettes when the Src oncogene is active (Fig. 3D), and (3) linear invadosomes in response to type I collagen fibres (Juin et al., 2012) (Fig. 3B–D). Furthermore, cells react differently to the substrate depending on its stiffness (Collin et al., 2006; Juin et al., 2012; Labernadie et al., 2014). For instance, a recent study has demonstrated that cells from the metastatic breast cancer line MDA-MB 231 preferentially form integrin-dependent actin dots on a high density of fibrillar collagen, which corresponds to compressed and fixed collagen I (Artym et al., 2015). Several other studies have demonstrated that cells preferentially form linear actin structures on fibrillar collagen I (Monteiro et al., 2013; Schachtner et al., 2013). Based on these findings, we propose that the resulting invadosome architecture might depend less on the particular cell type than on the experimental setting used for their observation. Invadosome plasticity has already been reported in certain cell types, such as osteoclasts, cancer cells, macrophages and endothelial cells (Destaing et al., 2003; Juin et al., 2012; Van Goethem et al., 2010). Osteoclasts are large monocyte-derived cells that can exhibit two different actin cytoskeleton organisations according to the differentiation state of the cell and the substrate (Saltel et al., 2008). Here, invadosome organisation changes during differentiation; initially, osteoclasts exhibit aggregates of invadosomes, before invadosome rosettes emerge from these aggregates during differentiation. At the end of differentiation, the invadosome rosettes expand to the cell periphery, fuse to each other and form a stable structure (Destaing et al., 2003). Moreover, when osteoclasts are seeded on mineralised matrices, the invadosome rosette is reorganised into a structure named the sealing zone that appears larger and denser, and that is dependent of the bone substrate (Saltel et al., 2004). In another example, depending on whether they are cultivated on two-dimensional (2D) or three-dimensional (3D) substrates, macrophages are able to extensively modify their overall cell shape, together with a total reorganisation of the actin cytoskeleton. Consequently, the number and the morphology of their invadosomes are strikingly different in 2D and 3D environments (Cougoule et al., 2010; Van Goethem et al., 2010). ECM matrix stiffness can also result in an increase in the number, size, stability and activity of invadosome structures (Alexander et al., 2008; Juin et al., 2013). Furthermore, a recent study has demonstrated that invadosomes are preferentially formed under the nucleus and that nucleus stiffness favours invadosome formation (Revach et al., 2015). These data suggest that invadosomes are also capable of sensing intracellular stiffness, such as that of a stiff organelle.

Moreover, different invadosomes organisation can be observed at a given time in a same cell. Indeed, in some cases, we observed some intermediate structures between linear and rosette organisation (F.S., unpublished data). These different examples

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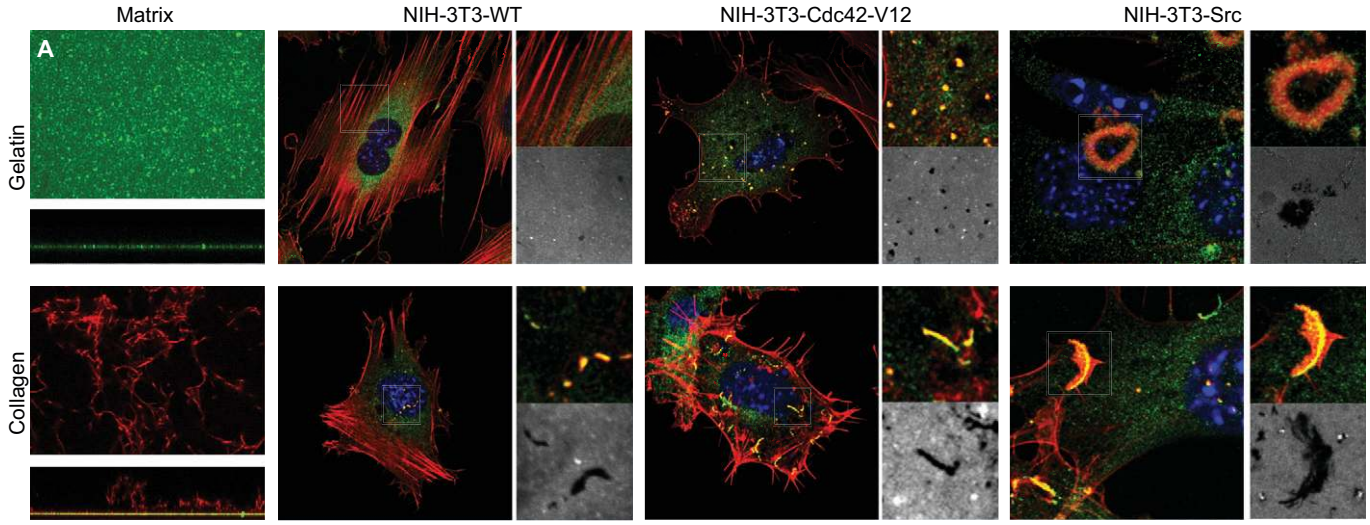

Fig. 3. Invadosome plasticity. (A) 2D and 3D confocal images representing a simple gelatin matrix in green (upper panel) and a mixed gelatin and collagen matrix (lower panel). Gelatin is in green and collagen is in red. (B) Wild-type (WT) NIH-3T3cells seeded on gelatin exhibit only stress fibres (upper panel) without any associated gelatin degradation (as seen by the grey colour in the inset), but when seeded on collagen form linear invadosomes (lower panel) that are associated with gelatin degradation (dark 'holes' in the bottom insets). (C) NIH-3T3 cells transfected with an active form of Cdc42 (Cdc42-V12) seeded on gelatin form active invadosomes that are organised in dots (upper panel and inset); the gelatin degradation pattern corresponds to the morphology of the invadosomes dots. Linear invadosomes are formed when the same cells are seeded on a collagen fibrils (lower panel); at this point, the gelatin degradation pattern appears linear (bottom inset). (D) NIH-3T3 cells constitutively expressing an active form of Src form invadosomes rosettes when seeded on gelatin (upper panel) with a corresponding degradation pattern. Linear invadosomes are induced when cells are seeded on collagen matrix (lower panel), and the gelatin degradation pattern follows the linear organisation. White asterisks show linear invadosomes on type I collagen. Actin is in red, Tks5 in green, nuclei in blue and gelatin in grey. Scale bars: 10 µm.

demonstrate that invadosomes are plastic structures that are highly adaptable to the matrix microenvironment.

Invadosomes *in vivo* – the beginning!

Since their discovery, invadosomes have been widely and mostly studied *in vitro*. It is only recently that work has aimed to provide evidence for their existence and functions *in vivo*. Identifying invadosomes *in vivo* in their natural environment is, however, challenging, mostly because the existing intravital imaging technologies are still suboptimal, especially in terms of the resolution available to simultaneously follow several molecular markers. Moreover, potent biochemical tools to demonstrate their relevance *in vivo* are still lacking. Thanks to the development of new microscope techniques, such as intravital multiphoton microscopy, it is now becoming possible to observe a cell within its surrounding matrix *in vivo* (Condeelis and Segall, 2003; Sahai et al., 2005; Wolf et al., 2003). Consequently, invadosomes are now being studied in different animal models, such as zebrafish, chicken, worm, frog and mouse, as well as within the context of both physiological and pathological processes (see Table 1). Below, we discuss the major recent findings that highlight the *in vivo* relevance of invadosomes.

In different animal cell models, 'invadosome-like protrusions' have been observed in cells crossing the basement membrane. For instance, one study has investigated the epithelial cell invasion within the developing intestine of zebrafish with a mutation in the myosin heavy chain 11 (*mlt*), which constitutively activates this protein, leading to a disrupted intestinal architecture (Seiler et al., 2012). Using time-lapse imaging, the authors showed that epithelial cells have actin-rich protrusions that are enriched in cortactin and Tks5. They also observed a weak expression of MT1-MMP at the basal area of the cell (Seiler et al., 2012). Thus, cells can successfully cross the basement membrane, most likely through a combination of a proteolytic activity and mechanical forces. The interaction between invasive cells and the basement membrane has also been studied in *Caenorhabditis elegans* (Hagedorn et al., 2013); here, actin-rich protrusions that breached the basement membrane were observed in the anchor cell, a single cell in the embryonic gonad that establishes the fate of the vulval precursor cells. However, even if these structures are somewhat similar to invadosomes with regard to their composition and structure, thus far, there is no evidence for their proteolytic activity. It should be noted though that, in a previous study, the same group had shown that a mutation of the *FOS-1* gene, which encodes for the MMP ZMP-1, reduces the invasiveness of the anchor cell, suggesting that a proteolytic activity is indeed involved (Sherwood et al., 2005). After breach of the basement membrane, the invadosome-like protrusions of the anchor cell disappeared. In this system, a single large protrusion was sufficient for invasion, which is mediated here by membrane displacement (Hagedorn et al., 2013). Taken together, these results might point to a new mechanism of invasion through basement membrane, which could combine protease activity and mechanical forces (Morrissey and Sherwood, 2015). Future work will be needed to determine whether these particular protrusions are indeed similar to the invadosomes described *in vitro*.

In vitro, after cytokine treatment, human umbilical vein endothelial cells are able to form invadosome rosettes that have been correlated with a degradative activity (Osiak et al., 2005; Tatin et al., 2006; Varon et al., 2006a). Using an *ex vivo* angiogenesis model, rosette structures that colocalised with cortactin in endothelial cells have been observed; these structures were associated with a decrease in laminin staining underneath, suggesting a degradation activity (Rottiers et al., 2009). More recently, similar invadosome rosettes have been identified in mouse angiogenic endothelium in response to VEGF-A treatment (Seano et al., 2014). Furthermore, the rosettes proved to be necessary for blood vessel branching and pathological angiogenesis. Moreover, integrin α6β1 was found to be required for the rosette formation and

Table 1. Overview of the studies describing invadosome-like structures *in vivo*

Animal model	Cell type	Markers	Microscopy	Microenvironment	Reference
Zebrafish	Intestinal epithelial cells	F-actin, Cortactin, Src, MMP14a	Time-lapse microscopy	Basement membrane	Seiler et al. (2012)
Nematode	Anchor cell	F-actin, PI(4,5)P2, Rac	Time-lapse microscopy	Basement membrane	Hagedorn et al. (2013)
	Anchor cell	F-actin, PI(4,5)P2, Cofilin	Confocal microscopy	Basement membrane	Hagedorn et al. (2014)
Xenopus	Rohon–Beard neuron growth cones	Cortactin and NCAM	Structure illumination microscopy (SIM)	n.d.	Santiago-Medina et al. (2015)
Chicken	Cancer cells	F-actin, Cortactin, Tks4, Tks5, MT1-MMP	Multiphoton intravital imaging, Confocal	Chorioallantoic membrane (CAM)	Leong et al. (2014)
Guinea pig	Lymphocyte and basophil	None	Electron microscopy	Vessel	Carman et al. (2007)
Mouse/Rat	Rat mammary adenocarcinoma cells (MTLn3)	N-WASP, Cortactin, Actin and Collagen degradation	Multiphoton Intravital Imaging, Confocal	Type I collagen	Gligorijevic et al. (2012)
Mouse	Breast cancer cell line (MDA-MB-231)	Cortactin, Tks5 and Collagen degradation	Multiphoton Intravital Imaging, Confocal	Type I collagen	Gligorijevic et al. (2014)
	Endothelial cells	F-actin, Integrin α6, cortactin	Confocal microscopy	Basement membrane	Seano et al. (2014)
	Smooth muscle cells	Tks5 and Cortactin	Electron microscopy	n.d.	Quintavalle et al. (2010)
	Osteoclast	None	Electron microscopy	Bone matrix	Masarachia et al. (1998)

n.d., not determined.

stabilisation. Although these structures expressed MT1-MMP, as shown *in vitro*, their ability to degrade the basement membrane *in vivo* remains unclear (Seano et al., 2014).

With regard to invasion of tumour cells into the matrix, protrusions adjacent to collagen fibres, macrophages and blood vessels have been found in a recent study (Gligorijevic et al., 2014). Here, the inhibition of MMPs with the drug GM6001 or a knockdown of Tks5, which colocalises to these invadosome-like structures, prevented their formation. The Tks5-containing protrusions were able to degrade type I collagen fibres as observed by immunofluorescence of primary tumour cryosections. In demonstrating the potential relevance of these protrusive structures *in vivo* (Gligorijevic et al., 2014), that study thus paves the way for future research efforts in identifying the relevance of invadosomal structures for tumour cell invasion, especially in the context of a disruption of the basement membrane.

Moreover, the role of the tumour microenvironment during metastasis has been described, in particular the role of macrophages. For instance, the presence of macrophages during intravasation has been shown to enhance the entry of tumour cells into the blood vessels (Roussos et al., 2011), and, furthermore, a paracrine signalling feedback between macrophages and tumour cells has been identified (Wyckoff et al., 2007). In addition, a further study has highlighted that the contact between macrophages and cancer cells increases the intravasation of MDA-MB-231 cells by enhancing the formation of invadosomes, both *in vitro* and *in vivo* (Roh-Johnson et al., 2014). However, another study from the same group suggests that tumour cell intravasation can be mostly attributed to macrophage-induced vascular permeability (Harney et al., 2015). Nevertheless, invadosome-like protrusions have been shown to facilitate tumour cell extravasation prior to metastatic growth in the chick chorioallantoic membrane model (Leong et al., 2014). Future work will be needed to determine whether circulating tumour cells exploit protrusive and degradative invadosomes to help them breach through the physical barriers that are imposed by basement membrane barriers.

In order to be able to confirm any observations of 'invadosome-like' protrusions *in vivo*, the minimal set of markers that is required to validate the existence of these invasion structures in a cell needs to be agreed on by the community (Di Martino et al., 2014). Ideally, a minimum of two independent components or features should be used to assess the presence of invadosomal structures *in vivo*. Moreover, recent discussions in the field have led to the consensus that visualising invadosomal structures at high-resolution would help in validating the physiological relevance of these structures, and/or their composition *in vivo*. For this purpose, access to and use of the newly emerging super-resolution imaging techniques will be instrumental. Importantly, recent developments in intravital correlative microscopy technologies, which, for instance, allow combining of dynamic intravital imaging of sub-cellular structures with the high-resolution capability of volume electronic microscopy, could help define the ultrastructure and thus the cytoskeletal components of invasive protrusions in order to study their subcellular composition in physiological and complex ECM (Karreman et al., 2014, 2016). We recently noticed that single tumour cells at the invasive front of the same tumour can exhibit protrusions with distinct morphological and ultrastructural features, which supports the idea that tumour invasion, whether it relies on the invadosome or not, is highly plastic (Karreman et al., 2016). These and other recent technological breakthroughs hopefully will help to validate the *in vivo* existence of invadosomes (Ellenbroek and van Rheenen, 2014).

Conclusions and perspectives

As outlined here, we propose that invadosomes should not be considered as a number of varying distinct types of structures, but rather that they are instead a single entity that is able to adapt to the cellular microenvironment. Indeed, for a given invadosome, its

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shape and molecular composition result from a combination of the different conditions encountered by the cell at a specific moment and at a specific location. Such a plasticity is, of course, necessary for a cell to be able to invade different types of tissues and matrices, and as shown in Fig. 3, cells from the same cell type can form all the different invadosome types previously described. Until recently, mainly owing to the matrix element for the study of invadosomes, only two main invadosomes shapes had been described, dots (separated or organized in aggregates) or rosettes. In the past few years, we demonstrated another type of organisation, the linear organisation of invadosomes along type I collagen fibrils.

Potentially, all cells are able to form invadosomes and degrade the ECM. Indeed, under physiological conditions, this property is an integral part of the function of some cells, at least at some stages during development. In pathological scenarios, such as during carcinogenesis, tumour cells can use this capacity to migrate and invade surrounding tissues as schematically outlined in Fig. 4. For example, tumour cells could use invadosomes to degrade the basement membrane surrounding the primary tumour to invade the connective tissue and to penetrate into the lymphatic or blood vessels, which corresponds to the intravasation phase (Fig. 4). Alternatively, cancer cells could form dot-like or linear invadosomes. Invadosomes could also have a role in neo-angiogenesis. In this context, endothelial cells could form invadosome rosettes to degrade the endothelium basement membrane, as well as potentially linear invadosomes to help the tip cell to invade the connective tissue (Fig. 4). Interestingly, tumour or endothelial cells have been shown to form invadosomes *in vitro*, and several studies describe such invadosome conformation *in vivo* (Gligorijevic et al., 2014; Seano et al., 2014).

Taken together, the studies discussed here suggest that an invasive capacity is a common property of all cells, but which is repressed in most cells in normal tissues. For example, under quiescent conditions, endothelial cells are unable to form invadosomes; however, following stress and/or a modification of their microenvironment, the very same cells form invadosomes.

However, several important questions remain, including what are the minimal stimuli necessary and sufficient for inducing invadosome formation? Are degradation-efficient invadosomes indeed present and required *in vivo*, and if so what is their morphology (i.e. do they form dots, linear structures, rosettes, any others or all of these)? The plasticity of invadosomes is evident.

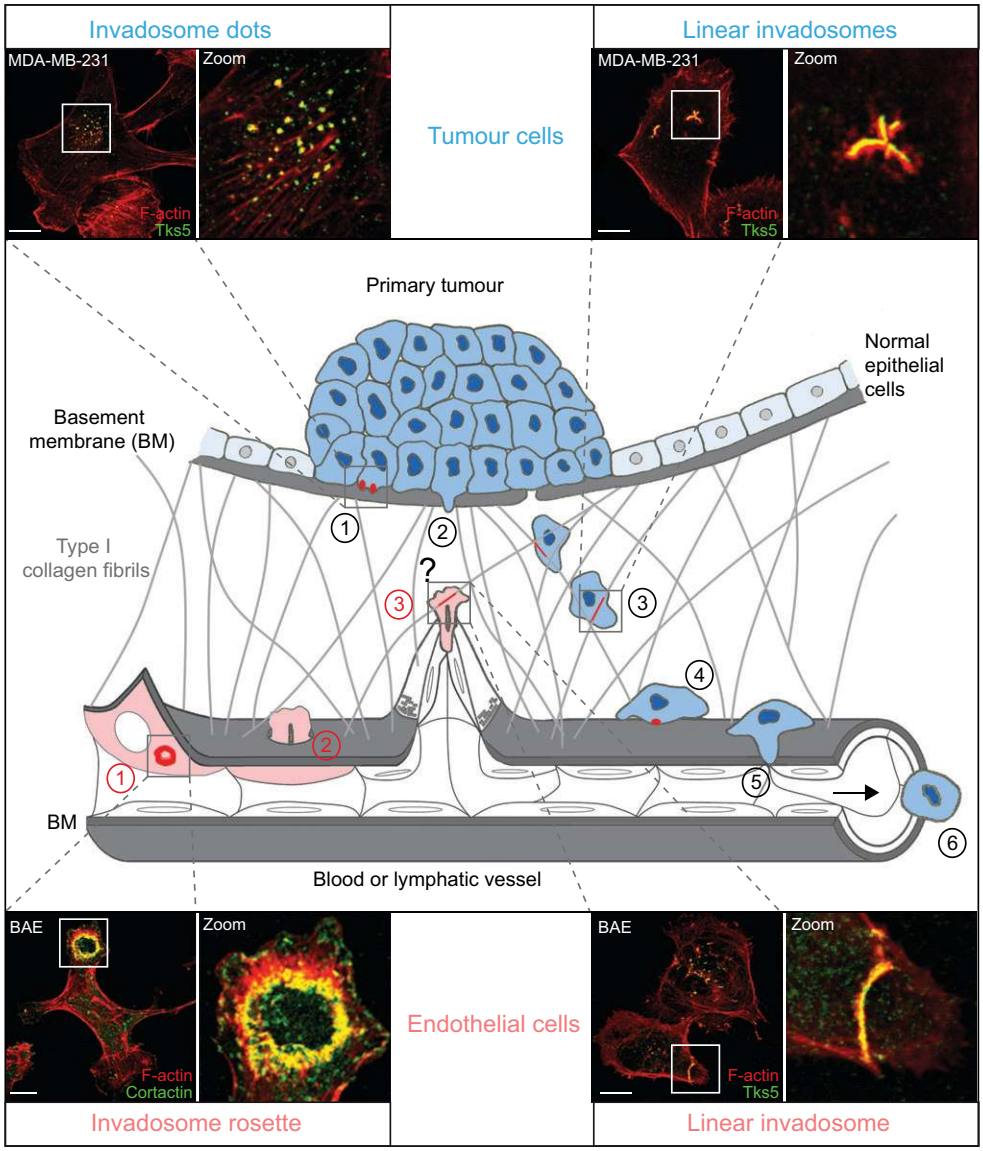

Fig. 4. Possible roles of invadosomes *in vivo*. The schematic representation in the centre of the figure illustrates the different steps of tumour cell invasion (blue) that have been associated with the presence of invadosomes (as shown by black numbers: 1, basal membrane degradation by tumour cell; 2, tumour cell protrusion; 3, potential linear invadosome formation into the stroma; 4, basal membrane degradation of the blood vessel; 5, tumour cell extravasation; 6, dissemination of tumour cell in the blood; and as shown by red numbers: 1, basal membrane degradation by endothelial cells; 2, endothelial cell protrusion; 3, potential linear invadosome formation in tip cell during neoangiogenesis). Endothelial cells are represented in red. Steps 1 to 3 (in red) refer to neo-angiogenesis steps with possible roles for invadosomes; invadosome rosettes or linear invadosomes might promote initiation and elongation of a new blood vessel, respectively. The upper panels show actin organisation of tumour cells that have been seeded onto either gelatin (left) or type I collagen (right). The confocal images in the lower panel are endothelial cells that have been seeded onto gelatin or type I collagen and form invadosome rosettes or linear invadosomes, respectively. F-actin is in red and Tks5 in green. Scale bars: 5 µm.

However, it is likely that the adaptation of a cell to its microenvironment complicates the identification of invadosomes *in vivo*. We thus should not search for a structure with only one specific feature, but for a multifaceted structure.

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Competing interests
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