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Regulation of invadopodia by mechanical signaling

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Abstract

Mechanical rigidity in the tumor microenvironment is associated with a high risk of tumor formation and aggressiveness. Adhesion-based signaling driven by a rigid microenvironment is thought to facilitate invasion and migration of cancer cells away from primary tumors. Proteolytic degradation of extracellular matrix (ECM) is a key component of this process and is mediated by subcellular actin-rich structures known as invadopodia. Both ECM rigidity and cellular traction stresses promote invadopodia formation and activity, suggesting a role for these structures in mechanosensing. The presence and activity of mechanosensitive adhesive and signaling components at invadopodia further indicates the potential for these structures to utilize myosin-dependent forces to probe and remodel their ECM environments. Here, we provide a brief review of the role of adhesion-based mechanical signaling in controlling invadopodia and invasive cancer behavior.

Keywords

invadopodia; mechanotransduction; signaling; adhesion; contractility; actin; invasion; extracellular matrix; proteinases; secretion

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Introduction

Cells sense the biomechanical properties of the ECM through interactions facilitated by matrix adhesions [1]. Intracellular adhesion proteins link ECM receptors to downstream force-sensing pathways, including non-muscle myosin II (NM II)-dependent contractility of adhesion-associated actin [2] and conformational changes of mechanosensitive proteins [3]. Changes in mechanical signaling pathways can alter cellular phenotypes and contribute to a number of diseases, including deafness, cardiac hypertrophy, and muscular dystrophy [4]. In breast cancer, increased ECM rigidity during tumorigenesis has been shown to drive a malignant phenotype through biomechanical adhesion signaling [5-8], including enhanced invasion and metastasis [8-11]. ECM rigidity changes in breast cancer are thought to occur as a result of a number of factors, including tumor cell packing, ECM deposition and crosslinking, and higher fluid pressures [12]. These factors are common features of many types of cancers [7, 12-14], and several other tumor types have also been quantitatively shown to have greater mechanical properties than neighboring normal tissues [15-17]. Recent studies have shown that mechanical factors alter the invasive properties of diverse cancer cell types in vitro [18-21] suggesting common rigidity-dependent regulatory pathways.

Proteolytic degradation of ECM promotes cancer cell invasion by allowing migration through dense cross-linked tissues such as the basement membranes that surround carcinomas and underlie blood vessels [22]. In addition, proteolytic remodeling of stromal collagen may allow collective migration of cancer cells through tissues [23]. In order to degrade ECM, cancer cells form actin-rich adhesive protrusions called invadopodia (Fig. 1) [24]. Invadopodia are cellular hotspots for secretion of matrix-degrading proteinases [25-27]; thus, formation of invadopodia greatly accelerates matrix remodeling. The ability of cancer cells to form invadopodia correlates well with their in vitro and in vivo invasive behavior [28-35]. In addition, upregulation in tumors of key invadopodia molecules, such as the matrix metalloproteinase MT1-MMP, and the actin assembly protein cortactin, are associated with poor patient prognosis [36, 37]. Similar structures called podosomes are formed in a variety of other cell types that need to remodel tissue or cross tissue barriers, including osteoclasts, endothelial cells, and macrophages [38].

In addition to invadopodia and podosomes, invadopodia-independent proteolytic degradation mechanisms have been described in normal and cancer-associated fibroblasts (CAFs) [39, 40]. Matrix degradation by fibroblasts at focal adhesions was regulated by signaling mechanisms that also control invadopodia (e.g. Src, FAK, p130Cas) [40]. However, invadopodia-independent plasma membrane sites were identified that do not depend on the critical invadopodia regulators Cdc42 or Src [39]. These data suggest some flexibility in the mechanisms controlling proteinase expression on the plasma membrane. In contrast, pancreatic CAFs expressing high levels of palladin have been shown to enhance invasion and metastasis of tumor cells through invadopodia-dependent ECM degradation [41]. While invadopodia appear to be the dominant mechanism used by invasive cancer cells to degrade ECM, further investigation is required to elucidate the role and regulation of proteolytic structures in tumor-associated stromal fibroblasts.

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Invadopodia formation and structure

Invadopodia are formed in response to signaling events that lead to dynamic branched actin assembly at membrane sites [25, 32, 42]. Shortly thereafter, proteinases are secreted and promote ECM degradation. MT1-MMP has been the most studied proteinase in invadopodia and is essential for degradation of in vitro crosslinked gelatin substrates [43-45] (Fig 1). However, many proteinases are secreted at invadopodia and could collaborate to promote degradation of ECM in tissues. These proteinases include MT1-MMP, MMP-2, MMP-9, seprase, cathepsin B, ADAM12, and uPAR [27, 28, 44, 46-50]. Some of these proteinases are also likely to activate latent ECM- and cell-associated growth factors [51-56].

By electron microscopy (EM), invadopodia are long, slender protrusions that are typically 50 nm in diameter and ~0.5-2 μm in length [30, 32, 57, 58]. While dynamic branched actin is found at the cortex and is an essential part of the formation process, the resemblance to filopodia by EM suggests that the actin found within the invadopodial protrusion is likely to be unbranched. Indeed, key filopodia proteins including fascin, Myosin X, mDia1, and fimbrin have been shown to be essential for invadopodia stabilization and elongation [32, 59, 60]. Thus, both the branched and unbranched actin nucleation machineries collaborate to form stable, active invadopodial protrusions.

Many signaling proteins localize to and regulate invadopodia formation and stability, including tyrosine kinases such as Src, EGFR, and Arg, adhesion proteins such as integrins, focal adhesion kinase (FAK), p130Cas, and integrin-linked kinase (ILK), and scaffold proteins such as Tks5 [24, 25, 61]. Many of these molecules also control podosome and focal adhesion formation and activity [62] (reviewed elsewhere in this issue). Src kinase is a particularly important regulator, as exemplified by the spontaneous formation of invadopodia-like structures in cells engineered to exogenously express constitutively active Src [63-65]. Given their similarities, invadopodia, podosomes, and Src-induced invadopodia-like structures are often referred to collectively as invadosomes [66].

ECM rigidity and cellular contractility control invadopodia formation and activity

One of the first indications that invadopodia might be involved in mechanical signaling came from our work demonstrating that ECM rigidity increases invadopodia numbers and activity [67]. At the same time, podosomes were found to exert shear stresses on flexible substrates and to participate in mechanosensing [68], suggesting general regulation of invadosome structures by substrate rigidity. Interestingly, we found that phosphorylated forms of the mechanosensing proteins p130Cas and FAK localize at actively degrading invadopodia, and their levels are reduced with inhibitors of nonmuscle myosin II (NM II) and myosin light chain kinase (MLCK) [67]. Overexpression of FAK and p130Cas also enhanced invadopodia activity on rigid but not soft substrates. While we did not find significant localization of phosphorylated MLC at invadopodia, in 40% of cells NM IIA was present in a ring-like structure around invadopodia [67]. Combined with the dependence of invadopodia activity on NM II activity, the localization data suggest regulation through the contractile machinery [67, 69]. Using tunable rigidity substrates as well as tissue-derived scaffolds, we further found that substrate rigidity controls invadopodia numbers and activity across a wide elastic modulus range [70]. The peak modulus for invadopodia-associated ECM degradation of our

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breast cancer model cell line was ~30 kPa which is in the rigidity range of tumor stroma [70]. In contrast, formation of MT1-MMP-positive membrane protrusions by several types of malignant cancer and fibrosarcoma cell lines has been shown to be inversely proportional to ECM rigidity [71]. The impact of these structures on ECM degradation was not quantitated; thus, further study is required to determine how they relate to invadopodia and invasion.

Cellular contractility plays an important role in mechanosensing by distinguishing the levels of ECM rigidity in the microenvironment and adjusting the amount of exerted cellular tension [72, 73]. Actomyosin-generated contractile forces [74] that are transmitted to the ECM can be measured as traction forces or stresses [75, 76]. Using traction force microscopy on substrates of different rigidities, the Parekh group found that the magnitude of overall cellular traction stress within a cell line is predictive of ECM degradation by invadopodia [77]. Across cell lines, the Reinhardt-King group found that the average cell traction stress produced on a substrate of a given rigidity correlates with the metastatic capacity inherent to a given cancer cell line [19]. These data suggest that cellular contractility is an important regulator of invasive behavior.

While the mechanical properties of the ECM may be one significant factor that influences cancer cell invasiveness, cells may exhibit varying responses to the same rigidity level. Thus, invadopodia dynamics may ultimately be dictated by the level of cellular force generation. The Varghese laboratory has recently shown that compressive traction stresses must reach a threshold level to induce invadopodia-like structures capable of ECM degradation [78]. These compressive stresses occurred at the protruding plasma membrane into the underlying substrate and were accompanied by shear and tensile stresses that together triggered proteolytic activity in response to mechanical resistance. These results complement our findings that NM II activity and augmented cellular traction stresses increase invadopodia numbers and induce more ECM degradation [67, 77]. They also suggest that ultimately it is the cellular response that matters, which could differ between cancer cells depending on the baseline signaling state and resultant contractility.

Adhesion components control invadopodia activity

Traction stresses generated by the actomyosin cytoskeleton are transduced to and from the ECM through adhesion complexes [75]. Multiple adhesion and contractility molecules localize to and around invadopodia, suggesting that some of the ECM rigidity response may occur directly at invadopodia [26, 62, 67, 70, 79]. While CD44 and β3 integrins have been found at invadopodia [80, 81], β1 integrins appear to be the predominant adhesion molecules found at or around their actin cores [49, 79, 82-85]. Although this localization could be due to the predominant use of fibronectin matrix for in vitro invadopodia assays, β1 integrins are frequently associated with cancer progression [49, 79, 82-85]. β1 integrins have been found to regulate invadopodia by forming signaling complexes with Src, EGFR, and/or FAK [82, 85]. In addition, β1 integrins have been shown to promote invadopodia maturation to actively degrading structures by mechanisms that include signal complex formation with ezrin at lipid rafts, regulation of actin dynamics by Arg, docking of the gelatinolytic enzyme seprase, and promotion of MMP secretion via integrin-linked kinase-IQGAP interactions (Fig. 2)

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[49, 79, 83, 84]. While these studies did not directly investigate mechanical signaling, many of these processes may be enhanced by ECM rigidity similar to the maturation of focal adhesions and podosomes that occurs in response to stiff matrices [68, 86]. For example, β1 integrins are known to regulate strength of cellular adhesions while β3 integrins reinforce force-dependent responses through talin-dependent mechanical signaling [87]. Similar mechanisms might operate at invadopodia, through the same molecules.

Similar to podosomes, invadopodia can organize adhesion proteins in rings around the actin cores [49, 67, 79, 88-90]. Using live cell imaging, Branch et al. showed that adhesion rings form shortly after formation of an invadopodial actin core [79]. Furthermore, β1 integrins found in invadopodial adhesion rings specifically enhanced the rate of MT1-MMP secretion [79]. Live cell imaging also revealed oscillations of actin at invadopodia that were paralleled by oscillations of GFP-paxillin-labeled adhesion rings [79]. Oscillatory behavior has been shown to be an important part of podosome mechanosensing and force generation [68, 91] and is regulated by actin polymerization and NM II activity [92, 93]. These data are consistent with a key role for adhesion-induced mechanical signaling in invadopodia maturation [49, 79, 83, 84].

Future Directions

Potential Methods for Studying Mechanical Signaling at Invadopodia

While multiple lines of evidence support the model that local mechanical signaling takes place at invadopodia [30, 49, 67, 70, 77, 79, 82-85, 94], further investigation is required to understand the molecular and biophysical mechanisms that regulate and respond to invadopodia mechanosensing. While studies with tunable rigidity substrates have been useful, more sophisticated methods to measure and manipulate force production at the subcellular level would allow closer investigation of local mechanical control of invadopodia. The standard method for measuring two-dimensional cellular forces is traction force microscopy in which stresses are calculated based on substrate deformations of elastic surfaces or pillars [75, 95, 96]. This method has been used to study podosomes; however, these structures can form large groups or rosettes which allows for easier measurements over a bigger area [68]. In contrast, invadopodia are often found isolated from each other making force generation more difficult to detect in such a small area [69]. However, traction force methods in which forces have been fitted to fluorescently-labeled focal adhesions [97] could be applied to invadopodia puncta in a similar manner. In addition, a novel method using the deformations in Matrigel networks has recently been developed to calculate the three-dimensional stresses generated by cells [78]. Such a method could potentially be further utilized to understand the role of adhesion-based mechanisms in regulating force-dependent proteolysis at invadopodia. In addition, other techniques adapted to study the mechanical nature of podosomes could be applied to invadopodia. For example, atomic force microscopy has been utilized to study the dynamics and stiffness of individual podosomes [92]. This technique was further developed into protrusion force microscopy in which protrusive forces exerted by individual podosomes during mechanosensing were measured based on deformations in polymeric films [91]. Such techniques could be complemented with advanced microscopy methods such as spinning disk confocal, FLIM, FRAP, and

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STORM with fluorescently tagged mechanosensitive proteins and/or tension biosensors [32, 93, 98, 99]. These techniques could be used to enhance our understanding of the molecular mechanisms that govern or respond to mechanical forces at invadopodia.

Understanding the Role of Local Forces at Invadopodia

One area that remains unclear is the role of different stress components during invadopodia mechanosensing and their part in invadopodia formation and/or maturation (Fig. 2). Focal adhesions generate traction forces via myosin contraction of actin stress fibers running parallel to the ECM surface that promotes their maturation [86, 100]. Similarly, invadopodia have been hypothesized to generate shear stresses through radial arrays of actin that surround their individual cores [62], similar to podosome rosettes [68]. Individual podosomes have recently been shown to exert protrusive or "pushing" stresses at their cores as a result of actin polymerization [91, 93], similar to what occurs at the leading edge of cells [101, 102]. These pushing stresses were accompanied by tensile or "pulling" stresses generated by actomyosin contractility surrounding the podosome cores [91, 93] suggesting that they occurred at the adhesion-based rings. This combination of pushing and pulling lead to oscillatory force generation at podosomes indicating a dynamic mechanism by which these structures are constantly mechanosensing their local microenvironment [91-93]. Similar 3D traction stresses may also regulate mechanosensing at invadopodia since they also exhibit oscillatory behavior (Fig. 2) [79]. However, the consequence of these forces is unclear. For example, the relationship between oscillatory forces, mechanotransduction signaling, and proteinase secretion is unknown.

Determining the Roles of Contractility Regulators in Invadopodia Dynamics

Another area of interest is how actomyosin contractility interfaces with other signals to control invadopodia. For example, molecules that regulate actin dynamics and cellular contractility such as the Rho GTPases act in a multitude of additional signaling pathways that may affect invadopodia independent of NM II-based force generation [26, 61, 103, 104]. For example, Rho-associated kinase (ROCK) is a classic activator of NM II via phosphorylation of MLC [4-6, 105]; however, ROCK has other downstream targets that regulate invadopodia such as LIM kinase, ezrin, and moesin [84, 89, 94, 106, 107]. While these are all actin regulators, they control other aspects including actin polymerization and linking actin to the plasma membrane. Therefore, it will be important to determine how contractile forces in cancer cells synergize with other signals to control invadopodia.

Developing New Models to Study Invadopodia

Validation of new findings regarding mechanical signaling by invadopodia requires models that capture the properties of the tissues encountered by invading cancer cells. The classic model for studying invadopodia has been the in vitro invadopodia assay, which relies on cancer cells plated on fluorescently labeled and cross-linked ECM for detecting degradation (Fig. 1) [108]. While this assay can be modified, e.g. by altering substrate rigidity using tunable synthetic substrates [76, 109], such systems do not fully recapitulate the tumor microenvironment. However, studying invadopodia in more complex environments has presented significant technical challenges given their size, dynamics, and lack of markers for ECM degradation in physiologic systems that can capably be monitored and imaged. Over

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the last several years, significant progress has been made to overcome some of these issues [110]. For example, Gligorijevic et al. recently identified cellular protrusions as invadopodia in vivo based on the presence and functionality of markers such as cortactin, Tks5, and MMP activity. Using high-resolution multiphoton microscopy of human breast carcinoma xenografts in SCID mice, they found that a variety of microenvironmental factors, including the density of collagen fibers, regulated the locomotion speed of invading cancer cells and resulting disease progression. In particular, slower moving cells required invadopodia in spatially distinct regions of the tumor microenvironment such as near collagen fibers and blood vessels [111]. Using photoconversion of cells expressing invadopodia and an MMP-activated fluorescent substrate, they demonstrated that invadopodia formation and ECM degradation directly correlated with subsequent intravasation and metastasis.

While clinical evidence and animal studies provide strong correlations between tumor ECM density and metastasis [8-11], other microenvironmental factors may significantly contribute to the phenotype of invading cancer cells and their expression of invadopodia [111]. Thus, invasion is influenced by matrix organization and pore size, hypoxia, extracellular vesicles and growth factors, and other cells in the microenvironment [27, 50, 54, 111-115]. In some cases, paradoxical relationships between ECM density and tumor aggressiveness exist. For example, unlike breast cancer, fibrosis appears to play an inhibitory role for pancreatic cancer [116-118]. In one study, depletion of myofibroblasts in a transgenic mouse model of pancreatic cancer resulted in increased invasion and decreased survival despite decreased tumor rigidity [116]. Alteration in immune cell phenotypes was found to account for the aggressive phenotype of pancreatic cancers lacking myofibroblasts, suggesting complex relationships in vivo. Therefore, further studies are required to elucidate how tissue rigidity impacts the aggressiveness of diverse cancer types and the role of invadopodia in that process.

Conclusion

Adhesions and mechanotransduction pathways control a variety of cellular phenotypes. The identification of invadopodia as mechanosensitive organelles suggests that the interface between adhesion signaling and additional molecular pathways can greatly enhance invasive behavior. Understanding that unique interface may help identify novel targets for therapeutic intervention. Future studies should combine a variety of state-or-the-art techniques and pre-clinical models to overcome previous biological and technological limitations and further elucidate molecular pathways. These models will be critical for providing the additional support necessary to link invadopodia to metastasis and explore the relevance of purported molecular mechanisms for therapeutic intervention.

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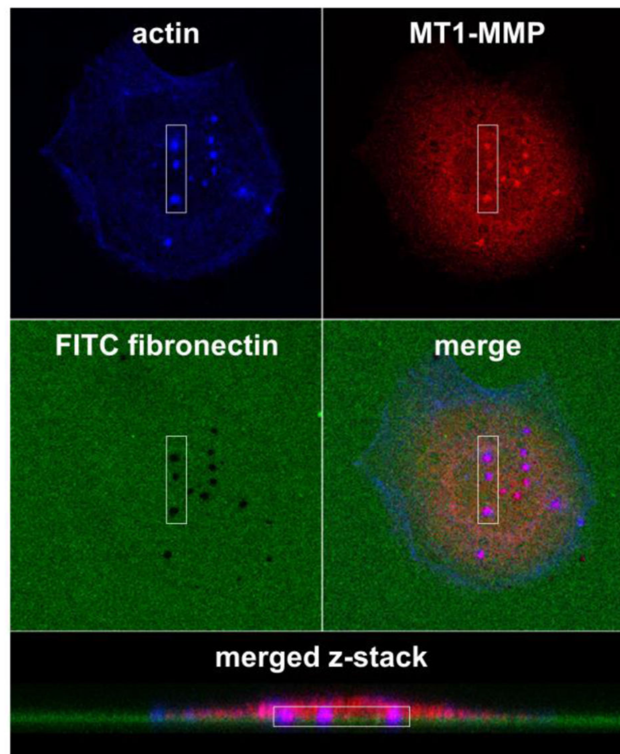

Figure 1.

Invadopodia are actin-rich proteolytic protrusions that are often identified through colocalization of markers with ECM degradation. The in vitro invadopodia assay typically consists of invasive cancer cells cultured on fluoroscently-labeled ECM, in this case FITC-fibronectin-coated crosslinked gelatin. After 6-48 h, the cells are fixed and stained for molecular markers of invadopodia including actin filaments, cortactin, Arp2/3 complex, Tks5, and/or MT1-MMP [44, 45, 76, 99, 108, 109, 119]. In this case, invadopodia are identified by colocalization (purple) of actin filaments (blue) and MT1-MMP (red) using confocal microscopy imaging. Mature invadopodia are further recognized by colocalization of invadopodia markers with areas of ECM degradation (black holes in the green FITC-labeled fibronectin).

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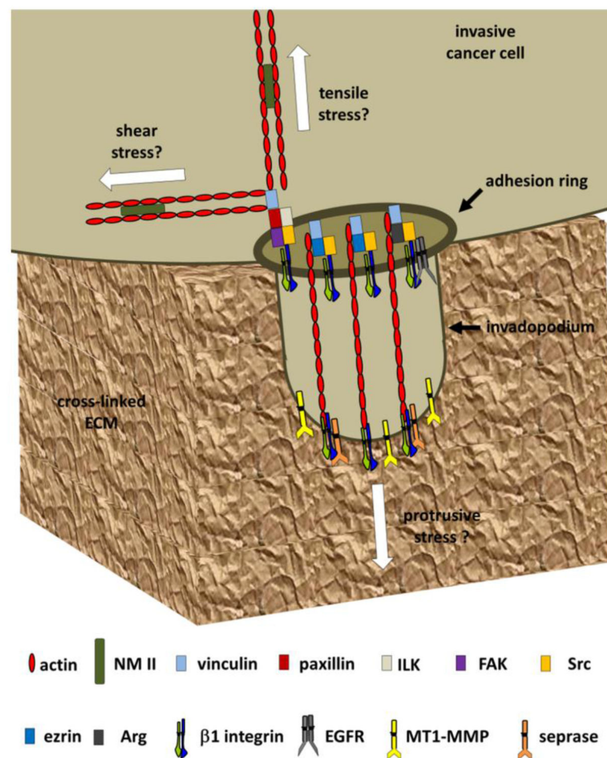

Figure 2.

Adhesion-based signaling in invadopodia. Signaling through β1 integrins at and/or around the actin cores of invadopodia regulates their maturation and ability to degrade ECM with proteases such as MT1-MMP and seprase [49, 79]. Adhesion rings are strongly correlated with invadopodia activity and are dependent on adhesion and signaling components such as vinculin, paxillin, ILK, and FAK [67, 79]. In addition, invadopodia activity is also regulated by the interactions of β1 integrins with other proteins and/or complexes that include ezrin, Arg, and EGFR [83-85]. Each of these pathways is regulated by Src kinase, which is thought to control early signaling cascades necessary for invadopodia formation [25, 61]. Adhesion rings may anchor and thus stabilize nascent invadopodia and generate NM-II generated shear and tensile stresses. Actin polymerization in the core may generate protrusive stresses, similar to those identified in podosomes [78, 91-93]. Periodic fluctuations in the stresses perpendicular to the ECM surface induce oscillations of invadopodia core and ring proteins [79, 91-93]. Together, these processes may be coordinated to constantly sense and respond to local ECM rigidity. Overall, adhesion based signaling in response to ECM rigidity is an important control point for invadopodia.