HHS Public Access

Author manuscript

Int Rev Cell Mol Biol. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Int Rev Cell Mol Biol. 2021 ; 360: 65–98. doi:10.1016/bs.ircmb.2020.09.004.

Actin dynamics during tumor cell dissemination

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Abstract

The actin cytoskeleton is a dynamic network that regulates cellular behavior from development to disease. By rearranging the actin cytoskeleton, cells are capable of migrating and invading during developmental processes; however, many of these cellular properties are hijacked by cancer cells to escape primary tumors and disseminate to distant organs in the body. In this review article, we highlight recent work describing how cancer cells regulate the actin cytoskeleton to achieve efficient invasion and metastatic colonization. We also review new imaging technologies that are capable of revealing the complex architecture and regulation of the actin cytoskeleton during motility and invasion of tumor cells.

1. Introduction

In metazoans, cell motility is required for key developmental processes, including gastrulation (Keller, 2005), neurulation (Theveneau and Mayor, 2012), the maintenance of tissue integrity and wound repair (Friedl and Gilmour, 2009), and immune cell trafficking (Friedl and Weigelin, 2008). Cell invasion is also conserved during embryonic development and homeostasis, as well as immune cell function (Medwig and Matus, 2017; Stuelten et al., 2018). The ability of a cell to move and invade is primarily dependent on the reorganization of the actin cytoskeleton, which requires spatiotemporal coordination of signaling pathways with actin regulatory and binding proteins (Blanchoin et al., 2014; Lauffenburger and Horwitz, 1996; Pollard and Cooper, 2009). Multiple human diseases and pathologies, including cancer cell invasion and metastasis, are associated with aberrant and deregulated cell motility and invasion (Hanahan and Weinberg, 2011).

The acquisition of motile and invasive phenotypes is a characteristic of aggressive tumors. Migration and invasion are needed for local invasion, intravasation into the vasculature, and extravasation at distant sites (Chaffer and Weinberg, 2011). The formation of actin-rich protrusions is a key feature of cancer cells that allows them to disseminate and colonize other organs (Bravo-Cordero et al., 2012). Thus, tumor cell motility and invasion are key rate-limiting steps during cancer progression and metastasis formation. Tumor cells are capable of activating different molecular mechanisms to remodel the actin cytoskeleton in order to leave primary tumors and travel to other organs (Bravo-Cordero et al., 2013a; Yamaguchi and Condeelis, 2007). From pseudopodia to filopodia and invadopodia

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protrusions, cancer cells display a repertoire of subcellular actin-rich structures that facilitate overcoming different barriers during tumor dissemination.

In this review, we address key modes of cancer cell motility and underlying signaling pathways, effects of the tumor microenvironment on cancer invasion, and recent technological advances that have been developed to visualize the invasion-metastasis cascade (Fig. 1).

2. Tumor dissemination and metastasis

In most epithelial cancers, tumor cells must acquire an invasive phenotype in order to escape the primary tumor and have the potential to invade locally, intravasate into the vasculature, survive circulation, extravasate into distant organs and colonize (Chaffer and Weinberg, 2011). The tumor microenvironment (TME) is a complex milieu of tumor cells, a dynamic extracellular matrix (ECM), and many stromal cells, including cancer-associated fibroblasts and immune cells (Fig. 1); in an orchestrated effort, components of the TME may act in concert to drive tumor cell invasion and metastasis formation (Clark and Vignjevic, 2015; Di Martino et al., 2019).

2.1 Epithelial-to-mesenchymal transition (EMT)

One mechanism by which epithelial tumor cells can become more invasive is by co-opting an epithelial-to-mesenchymal transition (EMT) developmental program (Dongre and Weinberg, 2019). An EMT induction results in the expression of core EMT transcription factors (*e.g.*, Twist, Slug, Snail, Zeb1, Zeb2) which upregulate genes that promote a mesenchymal-like cell migratory phenotype. An EMT or partial-EMT can be induced through TGF-β, Notch, and Wnt signaling pathways, and is often dependent on the secretion of specific chemokines and cytokines by the TME (Dongre and Weinberg, 2019).

Signaling through TGF-β1, which can be produced by tumor cells, cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells, and regulatory T cells (Tregs), as well as STAT3 signaling downstream of Il-6 and Il-23 secreted by leukocytes (Smith and Kang, 2013) has been shown to induce EMT. Tumor-associated macrophages, which are often found at the invasive front of tumors (Condeelis and Pollard, 2006) secrete pro-inflammatory cytokines such as TNF-α, which regulate the NF-κB pathway (Chen et al., 2018b). Multiple studies have demonstrated that EMT can be activated by TNF-α; TNF-α secreted by TAMs activated NF-κB signaling and stabilized Snail, which resulted in increased cancer cell invasion *in vitro* (Wu et al., 2009). TNF-α has also been demonstrated to upregulate Twist1 through NF-κB signaling in breast cancer cells (Li et al., 2012), induce EMT in renal cell carcinoma in a GSK3β-dependent manner (Ho et al., 2012), and stabilize Slug through NF-κB signaling in head and neck squamous cell carcinoma (HNSCC) (Liu et al., 2018a).

Induction of an EMT program can upregulate matrix metalloproteinases that are capable of degrading the basement membrane (Olmeda et al., 2007), which consists mainly of laminin and type IV collagen (Bosman et al., 1985). The Snail1 transcription factor is capable of inducing an invasion program dependent on MMPs; Snail1 induction increases both MT1-

MMP and MT2-MMP expression in breast carcinoma cells (Ota et al., 2009). In addition, a subset of invasive cancer cells can form invadopodia (see Section 4.3) structures, which are capable of recruiting MT1-MMP, MMPs, and ADAMs, which allow them to degrade the extracellular matrix (Eddy et al., 2017). EMT has been demonstrated to drive invadopodia formation in a Twist1-dependent manner (Eckert et al., 2011).

Recently, the concept of an EMT has been challenged in that it may not be necessary for metastasis formation (Aiello and Kang, 2019). Using an EMT lineage tracing system in the PyMT model of spontaneous breast cancer, lung metastases were shown to form when EMT was inhibited (Fischer et al., 2015), and the tracing system was validated using single-cell RNAseq (Lourenco et al., 2020). In a study on invasive ductal carcinomas, expression of E-cadherin promoted tumor cell survival and the establishment of metastases (Padmanaban et al., 2019); loss of E-cadherin is one of the hallmarks of an epithelial-to-mesenchymal transition (Dongre and Weinberg, 2019). In the KPC model of pancreatic ductal adenocarcinoma, loss of Snail or Twist did not have a significant reduction in metastases formation (Zheng et al., 2015), yet Zeb1 depletion in the same background did (Krebs et al., 2017), suggesting the requirement for EMT may vary based on the context (Aiello and Kang, 2019).

Additional factors in the tumor microenvironment can directly impact tumor cell dissemination; an important aspect of tumor biology is the mechanical properties of the surrounding ECM (Mohammadi and Sahai, 2018). It has been shown that *in vivo*, tumor cells move along highly aligned collagen fibers (Condeelis and Segall, 2003) to facilitate local invasion (Provenzano et al., 2006). Also, increased matrix stiffness can induce EMT through Twist1 activation (Wei et al., 2015).

2.2 Intravasation

After cancer cells invade locally, they can intravasate into the vasculature and travel through the hematogenous system or more rarely, through the lymphatic vasculature (Chiang et al., 2016; Olmeda et al., 2017) (Fig. 1). Capturing intravasation events *in vivo* is rare and challenging (Wyckoff et al., 2007); work using intravital microscopy combined with a mammary imaging window and photoconvertible Dendra2 to mark and track breast tumor cells demonstrated that vascularized regions had a greater number of photoconverted tumor cells lining up around the blood vessels, presumably intravasating into the vasculature, as characterized by more lung metastases (Kedrin et al., 2008). A proxy for intravasation events *in vivo* is to quantify circulating tumor cells in the blood. This type of analysis in a breast cancer model demonstrated that ERBB2 has a greater effect on intravasation than ERBB1; both are highly altered and aberrantly expressed receptors in aggressive breast cancers (Kedrin et al., 2009). Recent work in a PyMT model of breast cancer showed that $TIE2^{hi}$ macrophages were able to promote intravasation of tumor cells through the secretion of VEGFA, which resulted in transient permeability of blood vessels (Harney et al., 2015). The intravasation events characterized in this study were restricted to tripartite structures known as TMEMs (tumor microenvironment of metastasis), where a tumor cell, macrophage, and an endothelial cell are in direct contact with each other (Harney et al., 2015).

Additional studies on intravasation have used *in vitro* assays and microfluidic devices to characterize signaling pathways promoting intravasation. One mechanism by which tumor cells have access to the vasculature is through endothelial barrier impairment during tumor progression, which was demonstrated through macrophage-secreted TNF-α, which increased vasculature permeability and the rate of tumor cell intravasation (Zervantonakis et al., 2012). Invadopodia formation has also been linked with intravasation events; macrophages in direct heterotypic contact with breast tumor cells are able to induce global activation of RhoA signaling in tumor cells, resulting in tumor cells forming an increased number of invadopodia which are necessary for transendothelial migration (Roh-Johnson et al., 2014). A Notch1/Mena^{INV} signaling program has been demonstrated to regulate macrophage-induced invadopodium formation and transendothelial migration of breast cancer cells (Pignatelli et al., 2016).

2.3 Extravasation

Extravasation out of the vasculature has been visualized using *ex ovo* chicken embryos (Leong et al., 2014), zebrafish embryos (Berens et al., 2016), and tail vein injections in the mouse (Mohanty and Xu, 2010). One of the early studies in optically transparent zebrafish embryos showed that over-expression of Twist or VEGFA in highly invasive breast tumor cells increased the percentage of cells that were able to extravasate (Stoletov et al., 2010), and Twist expression induced a change in the mode of extravasation to β1-integrin independent (Stoletov et al., 2010). To examine the effects of inflamed neutrophils on tumor cell dissemination, LPS-stimulated neutrophils were co-injected with melanoma cells in zebrafish, resulting in increased extravasation (Chen et al., 2018a). Using an *ex ovo* chicken embryo model, human epidermoid cancer cells, as well as a series of other cancer cell lines were demonstrated to extravasate at endothelial junctions. Extravasation was dependent on invadopodia formation, as determined by localization of cortactin, Tks4, and Tks5, which are invadopodia components, using intravital imaging (Leong et al., 2014).

Tropism, or the homing of cancer cells to specific organs is often dependent on the tumor of origin; for example, the vast majority of patients with metastatic breast or prostate cancer have metastases in the bone (Weilbaecher et al., 2011). Cells in the bone secrete factors that attract cancer cells to the bone marrow, including RANKL, CXCL12, OPN, and BMPs (Jones et al., 2006; Obenauf and Massagué, 2015). Many breast cancer cells express EREG, MMP1, MMP2 and COX2 (Gupta et al., 2007), which allow them to selectively metastasize to the lung, as well as ANGPTL4 and SPARC (Padua et al., 2008; Tichet et al., 2015). Interestingly, COX2 and MMP1 signaling also allows breast cancer cells to overcome the blood brain barrier and form brain metastases (Wu et al., 2015), suggesting that some factors for tropism are organ-specific, and others are not as restrictive.

3. Cancer cell migration

3.1 Single-cell migration and the cell motility cycle

3.1.1 Mesenchymal motility—Within tumors, cancer cells can move as single, distinct entities or collectively as multicellular sheets or multicellular streams (Di Martino et al., 2019; Friedl and Wolf, 2010; Lintz et al., 2017; Roussos et al., 2011b). The tumor micro-

environment is a key component that regulates the different modes of tumor cell migration; for example, the ECM stiffness, density, and orientation that a tumor cell encounters is one parameter that determines whether cells move in a mesenchymal or amoeboid manner (Friedl and Wolf, 2010; Talkenberger et al., 2017). When cancer cells are in contact with stiff substrata, they are capable of adopting an elongated, mesenchymal-based mode of motility. The extension of a leading edge protrusion (lamellipodium in 2D or pseudopodia in 3D) is the first step of the cell motility cycle (Fig. 2). *In vitro*, flat, veil-like lamellipodia form as a result of membrane deformation due to force generated by dynamic actin polymerization (Bravo-Cordero et al., 2013a; Yamaguchi and Condeelis, 2007). As the lamellipodia extends forward and the actin network moves backward through retrograde actin flow, the cell body is able to make new attachments to the substratum through coupling actin stress fibers to adhesion receptors (*e.g.*, integrins). The cell body initially makes transient focal contacts with the substratum that can mature into focal adhesions, which are active signaling platforms that regulate mechanotransduction (Huttenlocher, 1995; Huttenlocher and Horwitz, 2011). As the cell is adhering in the front, it begins to disassemble focal adhesions in the rear and detach from the substratum. The cell body is able to translocate through the retraction force generated in the rear in a myosin II-dependent manner, which is regulated by Rho GTPase signaling (Friedl and Alexander, 2011).

In vivo, cancer cells can polarize (resulting in cellular asymmetry) and extend pseudopodial protrusions (Fig. 3) (Bravo-Cordero et al., 2012). Cancer cell polarization can be achieved through sensing external cues or gradients, including chemotactic (Roussos et al., 2011b), haptotactic (King et al., 2016), durotactic (DuChez et al., 2019), and galvanotactic (Huang et al., 2016), as well as mechanical and topographical constraints (Northcott et al., 2018). In response to these stimuli, polarized cancer cells are capable of activating signaling pathways that primarily converge on the Rho GTPase family to initiate actin polymerization and generate protrusions that extend outward from the leading edge, or front of the cell to facilitate cell movement (Bravo-Cordero et al., 2013b; Haga and Ridley, 2016; Lawson and Ridley, 2018; Yamaguchi and Condeelis, 2007).

In order for cancer cells to invade, they must remodel the extracellular matrix through expression of MMPs and other proteases (either cancer cell-intrinsic, or through stromal cells (Egeblad and Werb, 2002)) and in some cases, may recruit MT1-MMP, MMP2 and MMP9 through the formation of invadopodia structures, which are specialized F-actin-rich protrusions that degrade the extracellular matrix (Clark and Weaver, 2008; Clark et al., 2007; Eddy et al., 2017; Murphy and Courtneidge, 2011) (see Section 4.3). Recent work has shown that MMP activity can be regulated by cell density through an IL-6,8 paracrine loop (Jayatilaka et al., 2018), suggesting that MMP activity can be regulated locally through the homo-typic interactions between tumor cells.

3.1.2 Amoeboid motility—Another mode of single-cell movement is amoeboid motility, which is characterized by cells with a rounded morphology, and can present in multiple forms, including bleb-based (Petrie and Yamada, 2012). Bleb-based motility is movement that requires high levels of cell contractility, and resembles the motility of the single-cell organism *Dictyostelium discoideum* (Pinner and Sahai, 2008). Unlike

mesenchymal-like migration, blebbing motility is defined as protease-independent, and relies on the cell's capability to deform rapidly through dynamic alterations of the cortical actin cytoskeleton in the rear in a Rho-ROCK dependent manner (Sahai and Marshall, 2003). The high levels of contractility necessary for the cell to propel forward is dependent on the phosphorylation of myosin II-light chain (MLC2) via ROCK kinases, which are effectors of the Rho GTPases (Wilkinson et al., 2005). In addition, amoeboid motility usually occurs in areas with soft matrix, and amoeboid cells often have a decrease in integrin signaling and form weak adhesions (Brábek et al., 2010; Talkenberger et al., 2017). Interestingly, the use of protease inhibitors in HT-1080 fibrosarcoma cells and MDA-MB-231 breast carcinoma cells resulted in a transition from mesenchymal-to-amoeboid motility, rather than an abrogation of cellular invasion, demonstrating the plasticity of cancer cell movement (Wolf et al., 2003).

Cancer cells are able to interconvert between different motility patterns (Wolf et al., 2003). In triple-negative breast cancer cells, loss of the NEDD9 scaffolding protein resulted in more bleb-driven motility *in vitro*, including a loss of pFAK/pPaxillin mature focal adhesions, an increase in pMLC2, and a concurrent decrease in active Rac1 and increase in active RhoA (Jones et al., 2017). Similarly, in melanoma cells, bleb-based movement was driven through an active Rac GAP, ARHGAP22, which inactivated Rac signaling; conversely, mesenchymal-like motility was regulated by the NEDD9/DOCK3 complex which activated Rac signaling (Sanz-Moreno et al., 2008).

In mammary adenocarcinoma tumors *in vivo*, single-cell motility is characterized by the rapid movement of cancer cells that display an amoeboid morphology with the presence of F-actin-rich protrusions named pseudopodia at the leading front (Bravo-Cordero et al., 2012; Condeelis and Segall, 2003). These pseudopodia protrusions are characteristic of fast moving amoeboid cancer cells and are involved in chemotaxis toward blood vessels prior to intravasation (Condeelis and Segall, 2003).

3.2 Multicellular movement

In a 3D environment or *in vivo*, cancer cells encounter a complex microenvironment and are highly plastic in their motile behavior. Individual cancer cells are capable of moving in multicellular streams (Friedl and Alexander, 2011; Roussos et al., 2011a,b). In human orthotopic breast xenografts, cancer cells have been visualized to move in a multicellular stream (where a minimum of two cells follow each other in a directed manner) (Patsialou et al., 2013), or in a stream with host cells, such as tumor-associated macrophages (TAMs) (Patsialou et al., 2013). Co-migration of TAMs and breast cancer cells has also been demonstrated in a rat mammary adenocarcinoma model, and the transgenic PyMT spontaneous model of breast cancer. The interaction between these two cell types is dependent on an EGF/CSF-1 paracrine signaling loop (Wyckoff et al., 2004). $Mena^{INV}$, a splice isoform of Mena, an actin regulatory protein, is spontaneously upregulated in invasive carcinoma cells; expression of $Mena^{INV}$ in a rat mammary adenocarcinoma model promotes multicellular streaming between tumor cells, as well as co-migration between tumor cells and TAMs in an EGF/CSF-1 dependent manner *in vivo* (Roussos et al., 2011a). Intravital imaging of B16 F2 tumors shows tumor cells following each other on the same tracks, in a

multicellular stream. The streaming cells have increased SRF reporter activity (when compared to non-motile cells) (Manning et al., 2015); SRF is a master regulator of the actin cytoskeleton, and regulates the transcription of 200+ actin-related genes (Olson and Nordheim, 2010). Multicellular streaming has also been observed in a human orthotopic glioblastoma xenograft model, where tumor cells at the "invasive" margin between the tumor and brain parenchyma are capable of moving in succession, and overall, migrate with a lower velocity and increased persistence when compared to other motile tumor cells (Alieva et al., 2019). Intravital imaging of melanoma xenografts demonstrates that melanoma cells are capable of both single cell motility (Fig. 4A), as well as streaming, multicellular motility (Fig. 4B).

3.2.1 Collective cancer cell migration—Collective cancer cell invasion is another mode of tumor cell movement found in many cancers, including breast (Cheung et al., 2013), squamous cell carcinoma (Hidalgo-Carcedo et al., 2011), liver cancer (Han et al., 2019), melanoma (Hegerfeldt et al., 2002), colorectal cancer (Chung et al., 2016), and lung cancer (Kuriyama et al., 2016). Collective cancer cell migration is sheet or strand-like multicellular movement that requires cancer cells to maintain cell:cell cohesion mechanisms. In some instances, cells at the front of the strand polarize and become "leader" cells, followed by a stream of "follower" cells (Friedl et al., 2012). Invasive fibrosarcoma and breast cancer cells have been demonstrated to move collectively upon large-scale MT1-MMP mediated proteolysis in a spheroid invasion model; tracks were initially created by "leader" cells, followed by larger tracks created by multicellular invasion strands (Wolf et al., 2007). Using 3D organoid and *in vivo* model systems of luminal breast cancer, multicellular invasion strands were characterized by "leader" cells that were K14+, and preferentially turned on basal epithelial markers (Cheung et al., 2013). Cancer cells are highly plastic; breast cancer cells *in vivo* have been demonstrated to switch from collective to single cell migration through increased local TGF-β signaling (Giampieri et al., 2009).

The mechanisms underlying collective cancer cell migration are not well understood; there is some evidence that the Wnt/PCP (planar cell polarity) non-canonical Wnt pathway can be co-opted from developmental processes to promote collective cell migration in gastric, ovarian and melanoma cancers (VanderVorst et al., 2019). The DDR1 receptor has been demonstrated to be required for collective cell migration of A431 squamous cell carcinoma through regulation of the actomyosin network and interaction with the Par3/Par6 cell polarity complexes (Hidalgo-Carcedo et al., 2011). The tumor microenvironment has also contributed to collective cell migration signaling. Cancer associated fibroblasts (CAFs) are able to transmit force to human A431 squamous carcinoma cells and mediate collective cancer cell invasion in 3D through the formation of heterophilic N-cadherin/E-cadherin adhesions (Labernadie et al., 2017).

4. Actin structures in cancer cell migration

4.1 Lamellipodia

Lamellipodia (as well as pseudopodia in a 3D context or *in vivo*) are protrusive structures formed at the leading edge of cells that can drive cancer cell migration. The generation of

lamellipodia requires nascent branched actin polymerization in order to generate sufficient force to push the cell membrane forward; this occurs through multiple mechanisms, including *de novo* nucleation via activation of the Arp2/3 complex through nucleation-promoting factors (*i.e.*, WASP and WAVE proteins) and the generation of free barbed ends (polymerization competent-ends of F-actin) through cofilin severing of pre-existing filaments (Bravo-Cordero et al., 2013a; Yamaguchi and Condeelis, 2007). Upstream regulation of the actin machinery that form lamellipodia include activation of migratory signaling pathways through extracellular stimuli, which converge on the Rho GTPase signaling node (Ridley, 2015). For example, Rac1 can promote membrane ruffling through interaction with WAVE complexes via IRSp53 (Miki et al., 2000), resulting in Arp2/3-mediated actin polymerization (Ridley, 2015).

Lamellipodium-driven migration is regulated directly by intricate coordination of the Rho GTPases RhoA, Rac1 and Cdc42, as shown by FRET biosensor imaging studies (Machacek et al., 2009) (see Section 6.2). In the context of cancer, the use of an optogenetic system with a photoactivatable Rac1 biosensor in prostate cancer cells demonstrated that Rac1-dependent lamellipodium extension functioned downstream of active PI3K signaling (Kato et al., 2014). RhoA has also been shown to play an important role during lamellipodium protrusion formation. By using a RhoA biosensor in breast cancer, studies have shown that the activity of this GTPase is highly confined to the first micron of the leading edge where it mediates lamellar extension (Bravo-Cordero et al., 2013b). Work with Rho GTPase FRET biosensors also revealed that another isoform from the Rho subfamily, RhoC, displays a particular spatial activation during protrusion formation. RhoC is activated in areas behind the leading edge where it regulates cofilin phosphorylation to confine cofilin activity (Bravo-Cordero et al., 2011). These studies showed that RhoA and RhoC GTPases have a unique spatiotemporal activation pattern that is necessary in order to achieve efficient lamellipodium extension.

GTPase signaling and activation is dependent on cycling between GDP-and GTP- bound states. This process is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Haga and Ridley, 2016), many of which are mutated and aberrantly expressed in different cancer types (Porter et al., 2016). For example, P-rex1, a Rac1-specific GEF which can be regulated through PI3K-PI(3,4,5)P3 and GPCR signaling, has been demonstrated to promote invasion in melanoma in a Rac1-dependent manner (Lindsay et al., 2011), and is required for ErbB2-driven breast cancer cell migration (Sosa et al., 2010).

At the level of actin binding and regulatory proteins, there are changes in expression of many key actin regulators in multiple cancers, including the WASP/WAVE family (Iwaya et al., 2007; Kulkarni et al., 2012) and Mena proteins, amongst others (Gertler and Condeelis, 2011; Olson and Sahai, 2008; Yamaguchi and Condeelis, 2007). For example, in invasive breast cancer cells derived from rat mammary adenocarcinomas and the PyMT model of breast cancer, signaling through chemotactic factors, such as EGF, can directly affect lamellipodia formation by regulating the gene expression of actin nucleators, including several Arp2/3 subunits, as well as actin regulatory proteins that antagonize capping, including Mena (Wang et al., 2007). Mena, an Ena/VASP protein that binds the barbed ends

of actin filaments and delays termination by capping proteins, has splice isoforms with distinct functions in breast cancer cells (Gertler and Condeelis, 2011); the Mena11a isoform dampens growth-factor elicited lamellipodial protrusions (Balsamo et al., 2016), whereas the $Mena^{INV}$ isoform promotes lamellipodial protrusions (Hughes et al., 2015; Philippar et al., 2008). In invasive breast cancer cells, Lamellipodin, a binding partner of Ena/VASP proteins, is required for EGF-dependent lamellipodial protrusion and can promote 3D cancer cell invasion through specific interactions with Scar/WAVE and Ena/VASP complexes (Carmona et al., 2016).

4.2 Filopodia

Filopodia are thin projections that require the elongation of bundled, parallel actin filaments; they arise primarily from *de novo* actin nucleation by formins, or through an Arp2/3-mediated convergent elongation model (Gupton and Gertler, 2007; Jacquemet et al., 2015). In migrating cells, filopodia have been found at the leading edge, where they are able to emerge out of the lamellipodium meshwork downstream of Rho GTPase signaling, which results in the regulation of proteins including Ena/VASP (which are enriched at filopodia tips) (Lebrand et al., 2004) and IRSp53 (an effector of Cdc42 that can induce membrane curvature) (Disanza et al., 2013). Filopodia are bundled by actin bundling proteins, such as fascin or alpha-actinin, and are capable of extracellular sensing and cargo transport (Jacquemet et al., 2015).

Filopodia-like protrusions (FLPs) have been observed in mouse mammary carcinoma cells that have extravasated into the lung parenchyma. FLPs are regulated by Rif and mDia2 and are decorated with β1 integrin. FLP contact with the ECM initiates adhesion-dependent signaling and results in increased tumor cell proliferation (Shibue et al., 2012). By using quantitative microscopy, recent work showed that filopodia density increases as breast cancer progresses (Jacquemet et al., 2017). In addition, filopodia stabilization through the L-type calcium channel is required for directed migration and invasion (Jacquemet et al., 2016). In a separate study, it was also identified that upregulation of Myosin-X in p53-driven cancers is needed for invasion through the formation of filopodia (Arjonen et al., 2014).

4.3 Invadopodia

Invadopodia are F-actin-rich protrusive structures that are formed by invasive cancer cells in contact with the extracellular matrix. Invadopodia have proteolytic function and can focalize the secretion and accumulation of metalloproteinases, such as MMP2, MMP9 and MT1-MMP (Eddy et al., 2017; Jacob and Prekeris, 2015). The ability of invadopodia to degrade the ECM promotes local invasion of tumor cells, intravasation, and extravasation events (Bravo-Cordero et al., 2012; Gligorijevic et al., 2012, 2014; Leong et al., 2014; Roh-Johnson et al., 2014).

Invadopodia are induced by a variety of stimuli. Growth factors such as EGF (DesMarais et al., 2009) and TGF-β1 (Mandal et al., 2008) stimulate invadopodia formation in breast tumor cells. GABA and EGFR, which are chemotaxis receptors, are involved in invadopodia dynamics *in vivo* and can guide cancer cell extravasation and promote brain tropism in breast cancer metastasis (Williams et al., 2019). Recently, IKKε has been described as a

novel regulator of invadopodia formation and can promote metastasis in colorectal cancer (Liu et al., 2020). The tumor microenvironment can also regulate invadopodia; extracellular fibrillar collagen I was demonstrated as stimuli for cancer cells to form invadopodia in both 2D and 3D (Juin et al., 2012). Interestingly, collagen I induces invadopodia formation through the DDR1 collagen receptor in a kinase independent manner. The DDR1 receptor aligns along collagen I fibers, establishing linear invadosomes that recruit Cdc42 via the Tuba RhoGEF resulting in increased proteolytic activity (Juin et al., 2014). Other components of the extracellular matrix, including SERPINB5 and CSTB, can increase invadopodia formation and *in vivo* extravasation in pancreatic ductal adenocarcinoma (PDAC) (Tian et al., 2020). Adipocyte-derived lipid uptake by FATP proteins overexpressed in melanoma cells was able to induce invadopodia formation and drive melanoma progression (Zhang et al., 2018). Mechanosensing of the extracellular matrix can also form invadopodia, as invadopodia can contain integrin receptors (Mueller et al., 1999; Peláez et al., 2019) and CD44 (Petropoulos et al., 2018).

The formation of invadopodia occurs in steps, and is regulated temporally; briefly, invadopodium precursor structures assemble downstream of signaling cues (Beaty and Condeelis, 2014). The invadopodium precursor core is composed of cortactin, N-WASP, cofilin, and actin; invadopodium precursors are incapable of matrix degradation. Within seconds, Tks5 is recruited to the early invadopodium precursor where it stabilizes the structure. Subsequently, cortactin is phosphorylated and promotes the maturation of invadopodia, which endows them with the capability to polymerize new actin filaments and degrade the ECM (Eddy et al., 2017).

Rho GTPases, including Rac1, RhoA and Cdc42, have functional consequences during invadopodia formation (Beaty and Condeelis, 2014). Overexpression of the active form of Cdc42 and Rac1 induces invadopodia formation in cancer cells (Dutartre et al., 1996; Nakahara et al., 2003). RhoA drives invadopodium maturation (Bravo-Cordero et al., 2011; Sakurai-Yageta et al., 2008). Cdc42 is also a critical regulator of invadopodia dynamics, and affects invadopodium precursor assembly and maturation (DesMarais et al., 2009; Sakurai-Yageta et al., 2008; Yamaguchi et al., 2005; Eddy et al., 2017). A minimal signature to define invadopodia was proposed in 2014; actin structures that colocalize with Tks5 and the active form of Ccd42 are considered invadopodia (Di Martino et al., 2014).

Rho GTPases play an important role in regulating invadopodium dynamics. Work using Rho FRET biosensors showed that RhoC activation regulates cofilin activity at invadopodia (Bravo-Cordero et al., 2011). Use of FRET biosensor technology also demonstrated that Rac1 is required for invadopodium disassembly (Moshfegh et al., 2014) and Rac3 regulates integrin signaling at invadopodia and adhesion to the extracellular matrix (Donnelly et al., 2017). In relation to invadopodia formation, there is a small body of work describing GEF and GAP activity: one study has described how RhoC, which is important for metastasis formation (Clark et al., 2000), is spatially regulated at invadopodia by p190RhoGEF and p190RhoGAP (Bravo-Cordero et al., 2011). p190RhoGAP inactivates RhoC within the invadopodium core, and p190RhoGEF activates RhoC in areas surrounding the invadopodia (Bravo-Cordero et al., 2011). A few GEFs have been shown to be important for invadopodia function, including Vav1 (Razidlo et al., 2014), β-PIX (Donnelly et al., 2017; Md Hashim et

al., 2013), Fgd1 (Ayala et al., 2009), Frabin (Nakahara et al., 2003), Trio (Moshfegh et al., 2014) and SGEF (Goicoechea et al., 2017), as well as some GAPs, including p190RhoGAP (Bravo-Cordero et al., 2011) and ArhGAP12 in melanoma and breast cancer cells (Diring et al., 2019).

4.4 Focal adhesions

Focal adhesions are dynamic signaling nodes that connect the actin cytoskeleton directly to the extracellular matrix (Huttenlocher, 1995). The main adhesion receptors that link the ECM to actin stress fibers are integrins, which are bidirectional signaling molecules that can be activated in an “outside-in” or “inside-out” manner (Hynes, 1992). Integrins can be activated by binding to their respective ligands (*e.g.*, collagens, fibronectin), and recruit adaptor proteins, such as talin, kindlin (Sun et al., 2019) and paxillin (Turner, 2000), F-actin binding proteins (*e.g.*, vinculin, alpha-actinin), receptor tyrosine kinases such as FAK (Hanks et al., 1992) and Src (Schaller et al., 1999), as well as the many proteins that make up the “adhesome” (Horton et al., 2016; Zaidel-Bar et al., 2007).

Cells are capable of forming multiple types of ECM-adhesions, including focal complexes, classic focal adhesions, and fibrillar adhesions in a 2D setting (Geiger and Yamada, 2011), as well as cell-matrix adhesions in 3D (Geiger et al., 2009). At the leading edge of a motile cell in 2D (within 1–2μm), nascent adhesions, or focal complexes (FCs) can form underneath the lamellipodium (Geiger et al., 2001; Nobes and Hall, 1995; Geiger and Yamada, 2011). Although not fully characterized, the molecular composition of focal complexes contains a few hundred proteins, including integrins, actin binding proteins (*e.g.*, talin), and signaling molecules (*i.e.*, FAK) (Zaidel-Bar et al., 2003; Geiger and Yamada, 2011). Focal complexes have short lifetimes, and can either disassemble rapidly or mature into focal adhesions. Focal adhesions, which are more elongated than focal complexes, remain primarily under the lamella (>2μm from lamellipodial tips) at the ends of stress fibers (Geiger et al., 2009; Geiger and Yamada, 2011). The maturation of focal complexes into focal adhesions requires tension and force, through actomyosin contractility (Choi et al., 2008; Giannone et al., 2007), tyrosine phosphorylation of certain proteins (*i.e.*, paxillin), alterations in protein composition through recruitment of scaffolding proteins and increased adhesion-based signaling (Geiger et al., 2009; Geiger and Yamada, 2011). Rho GTPase proteins have a role in the formation of ECM-adhesions; Rac1 can control focal complex formation, and RhoA has a role in the maturation of focal adhesions (Parsons et al., 2010). Force generation can convert focal adhesions into fibrillar adhesions, which are mainly composed of α5β1 integrin and tensin, and regulate processes such as fibronectin fibrillogenesis (Danen et al., 2002; Zamir et al., 1999; Geiger and Yamada, 2011). Focal adhesion turnover, which can regulate cell migration, requires Src activity and the phosphorylation of FAK (Wozniak et al., 2004).

Cell-matrix adhesions have also been observed in 3D, which behave differently than adhesions in 2D. In cell-derived matrix, fibroblasts were able to make adhesions that were α5 integrin and paxillin positive (Cukierman et al., 2001). Breast epithelial cells in an attached 3D collagen gel are able to form small 3D adhesions with phosphorylated FAK Y397, whereas the same epithelial cells in a floating 3D matrix have adhesions that are

absent of FAK phosphorylation (Wozniak et al., 2003); thus, 3D adhesions are mechanosensors that behave differently based on the ECM rigidity.

Cancer associated fibroblasts (CAFs) are key regulators of ECM deposition and remodeling during tumor progression, and are involved in paracrine signaling with tumor cells (Attieh and Vignjevic, 2016). In CAFs, Hic-5 was shown to promote the formation of fibrillar adhesions through interaction with tensin1, which are conserved in 3D cell-derived matrices (Goreczny et al., 2018). Interestingly, during breast cancer cell invasion, FAK, a key kinase at focal adhesions, differentially regulates tyrosine phosphorylation at focal adhesion and invadopodia components (Chan et al., 2009), suggesting there may be crosstalk between the signaling pathways that regulate the formation of actin-rich structures during cancer cell invasion.

5. The cell cycle and cancer cell invasion

In the hallmarks of cancer, a deregulated cell cycle state and cancer cell invasion have been regarded as distinct programs (Hanahan and Weinberg, 2011); however, recent evidence suggests that there is a closer link between regulators of proliferation and invasion during cancer progression than previously thought (Kohrman and Matus, 2017). In breast carcinoma cells, it has been elucidated that invadopodia preferentially form in the G1 phase of the cell cycle (Bayarmagnai et al., 2019). p27, a cell cycle inhibitor that binds to Cdk-cyclin complexes in the nucleus, is able to regulate tumor cell invasion when it mislocalizes to the cytoplasm (Chu et al., 2008). In addition, p27 has been demonstrated to localize to invadopodia (Bayarmagnai et al., 2019; Jeannot et al., 2017) and regulates its activity through a Rac1-PAK1-cortactin signaling axis (Jeannot et al., 2017).

In melanoma, tumor cells are able to switch between a high-proliferative/low invasive state to a low-proliferative/high invasive state, known as phenotype switching, as the disease progresses (Arozarena and Wellbrock, 2019). Tumor cells expressing high levels of the MITF transcription factor, low levels of the Axl receptor and the associated transcriptional program remain in the high-proliferative/low invasive state; a switch to an Axl high and MITF low state shifts cells into a high-invasive/low-proliferative program (Rambow et al., 2019). The reduction of transcription factor MITF has been shown to induce a G1 cell cycle arrest through p27, and concurrently leads to the downregulation of Dia1 and promotes ROCK-mediated invasion (Carreira et al., 2006). In the PyMT model of breast cancer, loss of p21CIP1 suppressed invasion and increased cell proliferation (Qian et al., 2013), suggesting that p21CIP1 may mediate switching between proliferation and invasion.

6. Imaging advances and future directions in studying tumor cell invasion

6.1 Single-molecule superresolution imaging

The advances in the study of the actin cytoskeleton have been driven by the implementation of different high-resolution imaging techniques. Actin-rich structures are complex subcellular entities that contain several actin regulatory molecules. Recent proteomics studies (Attanasio et al., 2011; Ezzoukhry et al., 2018) have elucidated the composition of invadopodia and invadosomes; however, these techniques are limited in that the spatial

distribution of the components are unable to be characterized. Recently, the development of superresolution microscopy has revealed the organization of structures such as focal adhesions and podosomes (matrix-degrading protrusions similar to invadopodia formed in cells with a monocytic lineage; Linder and Wiesner, 2015).

A seminal study from the Waterman lab revealed the supramolecular organization of focal adhesions using superresolution microscopy, specifically PALM (Kanchanawong et al., 2010). This study showed that focal adhesions are multilaminar structures formed by three layers: an integrin layer, a force transduction layer, and an actin layer. PALM microscopy (Stubb et al., 2019) has been utilized to show the architecture of focal adhesions of stem cells, demonstrating that the organization of cornerstone adhesions and central adhesions are different at the nanoscale level; similar superresolution techniques have been used to image podosomes (Cox and Jones, 2013).

PALM, STORM, and other single molecule superresolution techniques can be applied to study the spatial organization of lamellipodium and invadopodium structures in cancer cells. MDA-MB-231 triple-negative breast cancer cells plated on a gelatin matrix were imaged with both widefield fluorescent microscopy and direct STORM (Fig. 5, left and middle panel); the use of dSTORM greatly increases the image resolution of subcellular structures and provides more detailed structural information. As shown in Fig. 5, the lamellipodium of cancer cells show a distinct localization of cortactin and F-actin at the leading edge. Interestingly, dSTORM imaging of invadopodia reveals complex spatial distribution of cortactin and F-actin, where F-actin is strongly enriched in the invadopodia core and cortactin molecules are scattered throughout the invadopodia structure (Fig. 5).

6.2 FRET-based imaging

To analyze signaling pathways of tumor cell motility, FRET-based imaging of Rho GTPases has been utilized both *in vitro* and *in vivo* and provides spatial information on Rho GTPase signaling and activation. Single-chain FRET biosensors, a more recent development in the field, consist of an N-terminal GTPase effector fragment, two fluorophores (an acceptor and donor FRET pair) separated by a linker, and a C-terminal GTPase (*e.g.*, RhoA) (Donnelly et al., 2014; Mondal et al., 2020). When the GTPase is active (GTP-bound), it binds to the GTPase effector, resulting in the FRET pair coming into close proximity of each other and increasing the FRET signal (the donor fluorophore emission overlaps with the acceptor fluorophore excitation). Additional modifications of this technology include a near-IR FRET pair that allows for compatible imaging with other FRET-based biosensors (including CFP-YFP FRET pairs), as well as potential usage *in vivo* due to the optimal properties of near-IR fluorophores for deep imaging (Shcherbakova et al., 2018). In this particular study, the use of a near-IR Rac1 biosensor with a RhoA CFP-YFP FRET biosensor revealed that antagonistic RhoA and Rac1 activity in motile cells is dependent on ROCK signaling (Shcherbakova et al., 2018).

FRET-based imaging allows for spatial localization of Rho GTPase activity at a subcellular level and links it directly to tumor cell motility and invasion. For example, FRET biosensor technology has demonstrated that macrophage-tumor cell contact increases RhoA activity in tumor cells to promote invadopodia formation and intravasation (Roh-Johnson et al., 2014),

whereas Rac1 activity can increase as invadopodia structures disassemble (Moshfegh et al., 2014). FRET biosensors are now being utilized *in vivo* to analyze RhoA activity in invasive breast and pancreatic cancer (Nobis et al., 2017); further exploration with FRET biosensors *in vivo* will be essential to elucidate signaling mechanisms activated during invasion and metastasis formation.

6.3 Intravital imaging and the tumor microenvironment

Techniques used to image *in vivo*, such as two photon intravital imaging and lattice light-sheet microscopy, are revealing the dynamics of tumor cells at the single-cell level during the metastatic cascade. Two-photon microscopy has visualized cancer cell motility in tumors (reviewed in Mondal et al., 2020), and more recently, behaviors of the surrounding microenvironment (reviewed in Di Martino et al., 2019). For example, the extracellular matrix has a key effect on modes of tumor cell migration; a combination of intravital two-photon imaging and computational modeling was used to delineate how tumor cells move based on what extracellular matrix structures they encounter (Tozluoğlu et al., 2013). Immune cells within the TME have also been characterized with two-photon imaging. Longitudinal studies using two-photon microscopy of tumor-associated macrophages in glioblastoma (GBM) has clearly defined two distinct types of TAMs, brain-resident microglia and bone marrow-derived macrophages, that are demonstrated to have distinct migratory behaviors (Chen et al., 2019).

Recently, the development of lattice light-sheet microscopy has allowed for imaging of tumor cell extravasation events. Lattice-light sheet imaging of zebrafish xenografts with labeled vasculature can capture the dynamics of cancer cells during extravasation with high temporal resolution in 3D (Liu et al., 2018b). Further studies using new tools of high-resolution imaging will help to illuminate the interplay between the TME and cancer cells, and how the TME affects tumor cell motility and invasion.

7. Conclusion

Understanding the dynamics of the actin cytoskeleton will help to develop targeted therapeutics that may prevent the dissemination of cancer cells and metastasis formation. The application of superresolution microscopy to investigate the macromolecular organization of invasive structures will provide valuable information about the spatial and temporal formation of invadopodia and pseudopodia and how the different components organize. We can envision that drugs that perturb the spatial organization of these molecules may interfere with the function of these actin-rich structures and may prevent the invasion and migration of cancer cells. As more work in the imaging field is developed, our understanding of actin dynamics at actin-rich structures will reveal possible candidates and additional signaling pathways to target during tumor cell dissemination.

Acknowledgments

We would like to thank Jill Gregory for her illustrations (Figs. 1–3), Linnea Olofsson for the acquisition and analysis of the widefield and STORM images (Fig. 5), and the Microscopy CoRE facility at Mount Sinai. This work was supported by a Susan G. Komen Career Catalyst Research Grant (CCR18547848), an NCI Career Transition Award (K22CA196750), an NCI R01 (CA244780), the Schneider-Lesser Foundation Award, a Stony Brook-Mount

Sinai pilot award, an ACCRF award (to J.J.B.C); the Tisch Cancer Institute NIH Cancer Center grant (P30 CA196521). Chandrani Mondal received support from an NIH T32 CA078207 Training Program in Cancer Biology.

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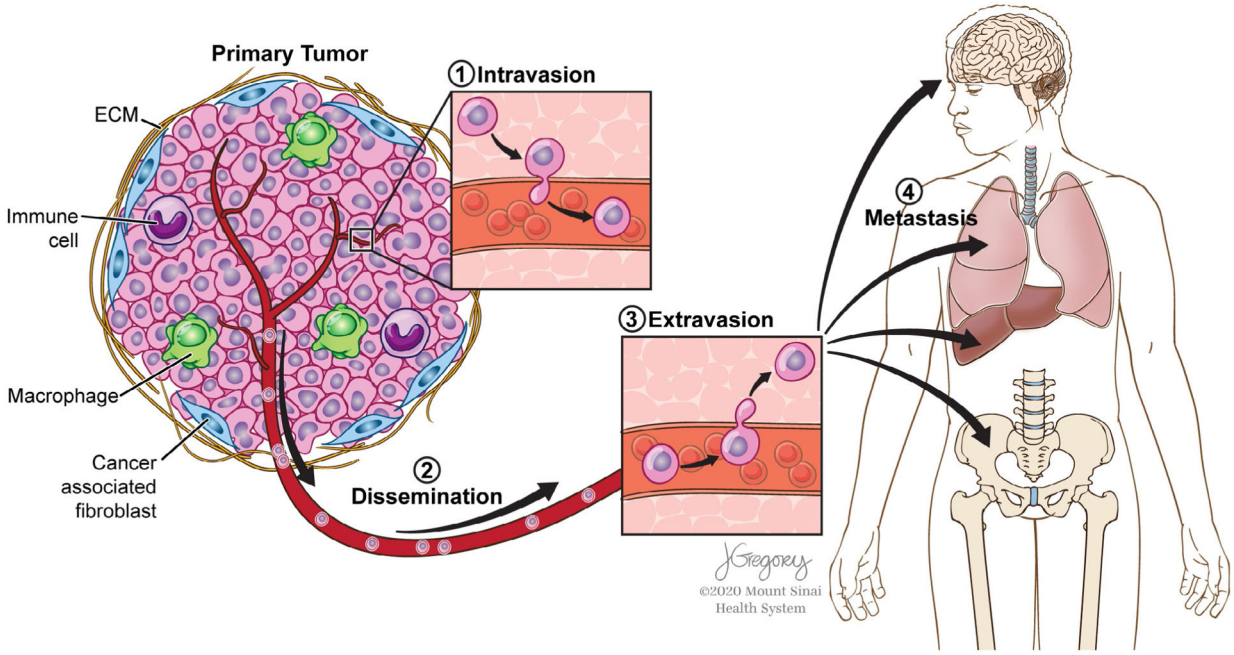

Fig. 1.

Invasion-metastasis cascade. Primary tumors have a complex tumor microenvironment (e.g., immune cells, cancer-associated fibroblasts, the extracellular matrix) that plays a dynamic role in affecting tumor cell dissemination and how tumor cells intravasate into the vasculature (1), disseminate through the circulatory system (2), extravasate out of the vasculature (3), and colonize distant organs (4).

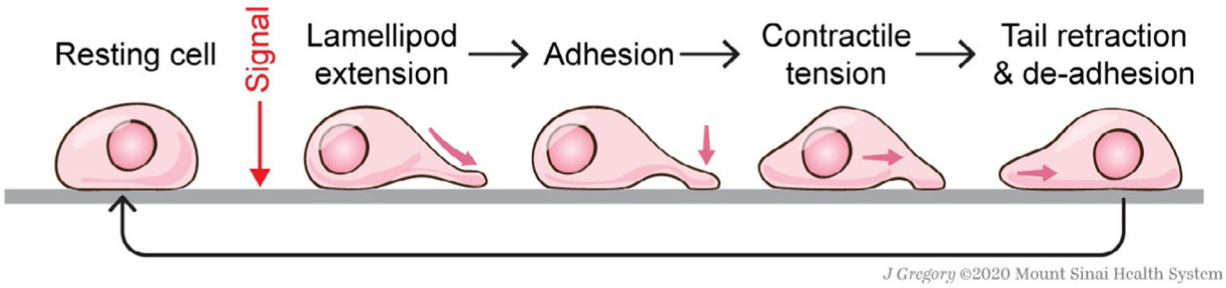

Fig. 2.

Cell motility cycle. Upon integrating signaling cues, a resting cell can form lamellipodia, or protrusive actin-rich structures, and adhere to the substrata with nascent adhesions. In order for the cell body to translocate, the cell experiences contractile tension and rear detachment through de-adhesion.

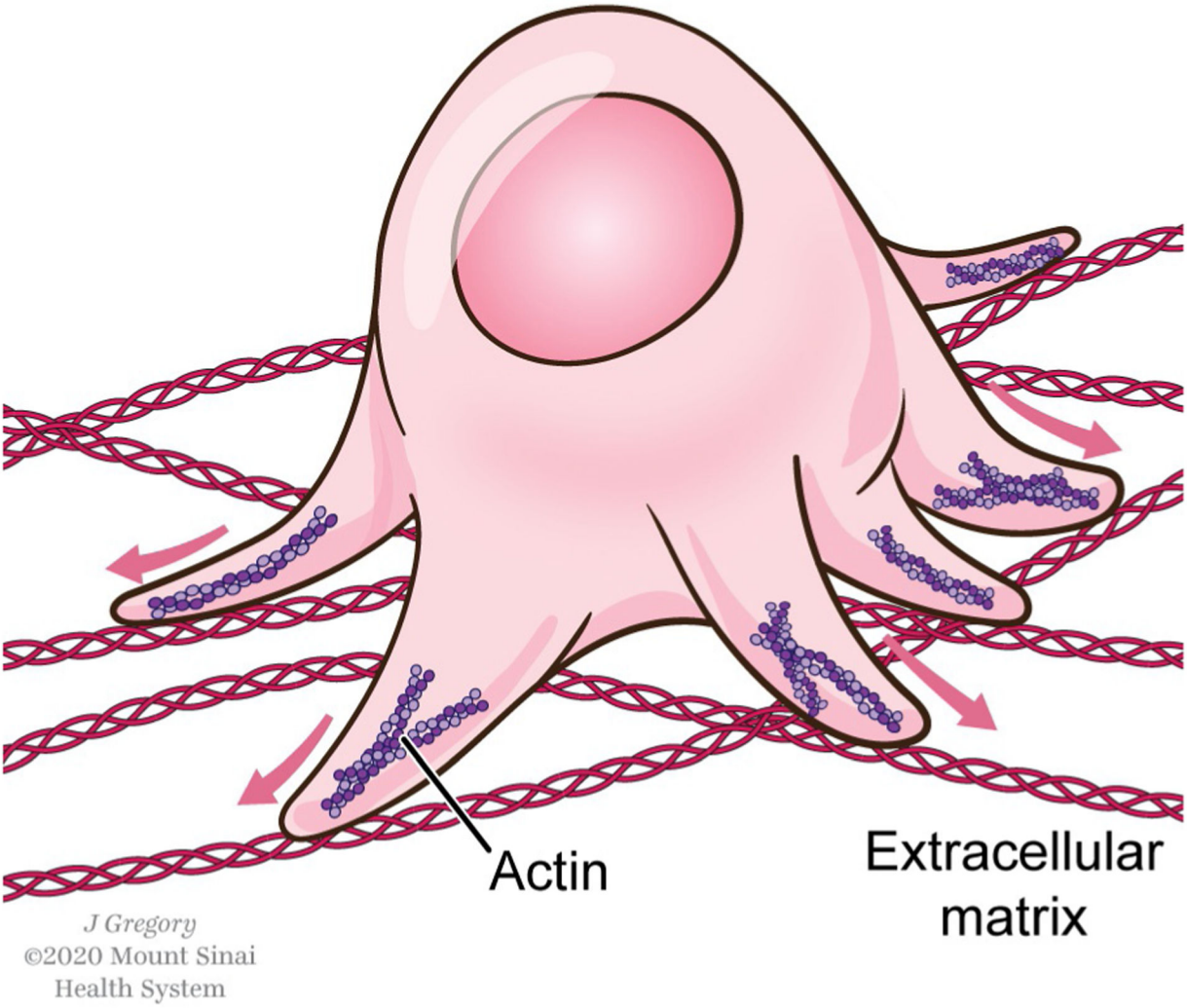

Fig. 3.

Tumor cell in a 3D context. In the tumor microenvironment, cancer cells encounter a complex extracellular matrix, and are capable of forming different types of protrusive structures, including pseudopodia.

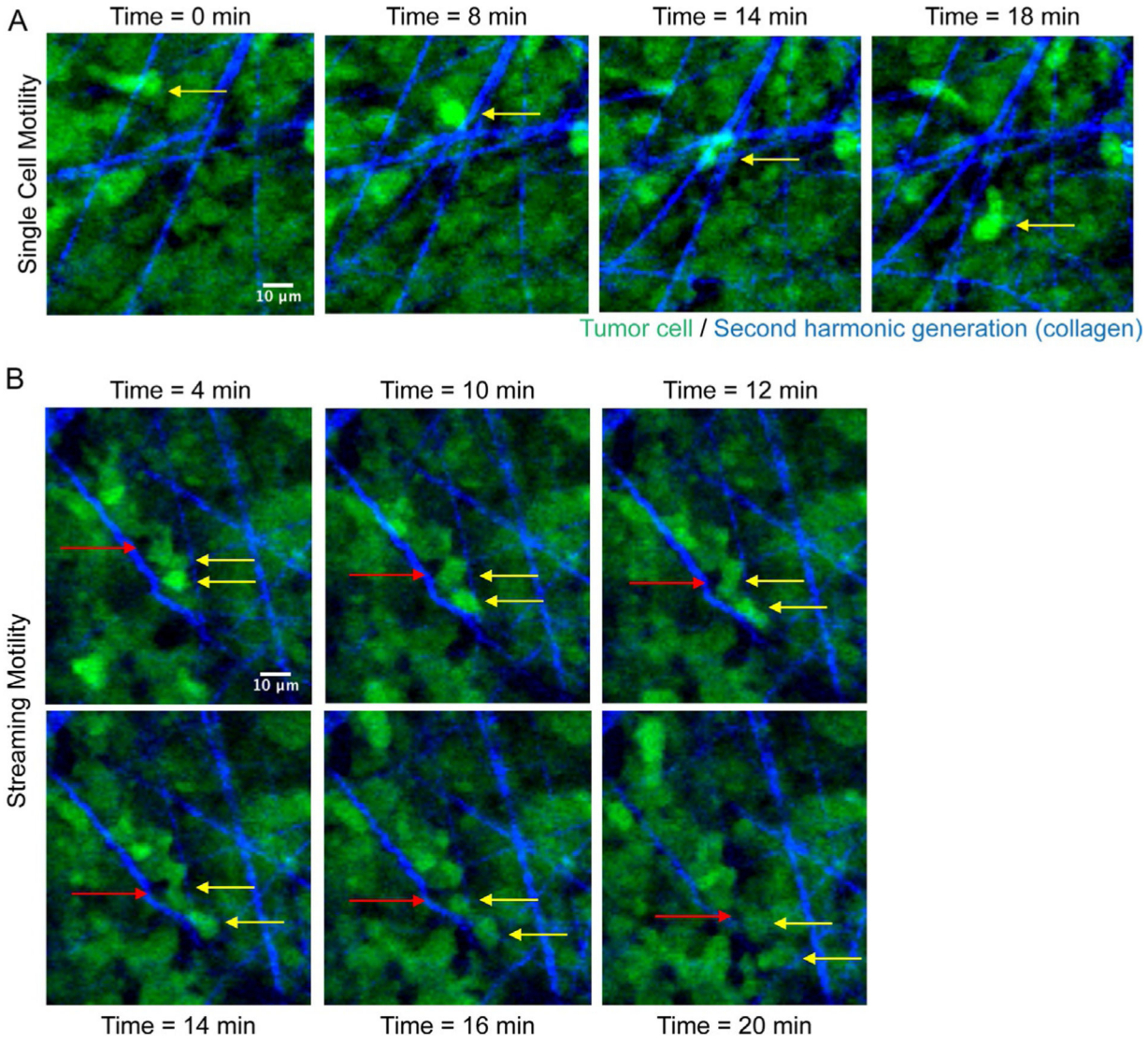

Fig. 4.

Intravital imaging of a melanoma xenograft. Two-photon imaging of tumor cells (in green) and second harmonic generation of fibrillar collagen (in blue). (A) Yellow arrow points to a single cell moving over time (indicated above each panel). (B) Example of streaming motility; yellow arrows point to cancer cells following each other, and red arrow points to another cell type in the tumor microenvironment moving within the multicellular stream. Scale Bar: 10μm. SK-Mel-147 GFP-labeled melanoma cells were injected subcutaneously in 6-week old female nude mice and tumors were allowed to grow up to $1cm^3$. Intravital imaging of the primary tumor was performed as in Patsialou et al. (2013) and collagen fibers were visualized by second harmonic generation. Images were acquired every 2min for 30min, with a step-size of 5μm.

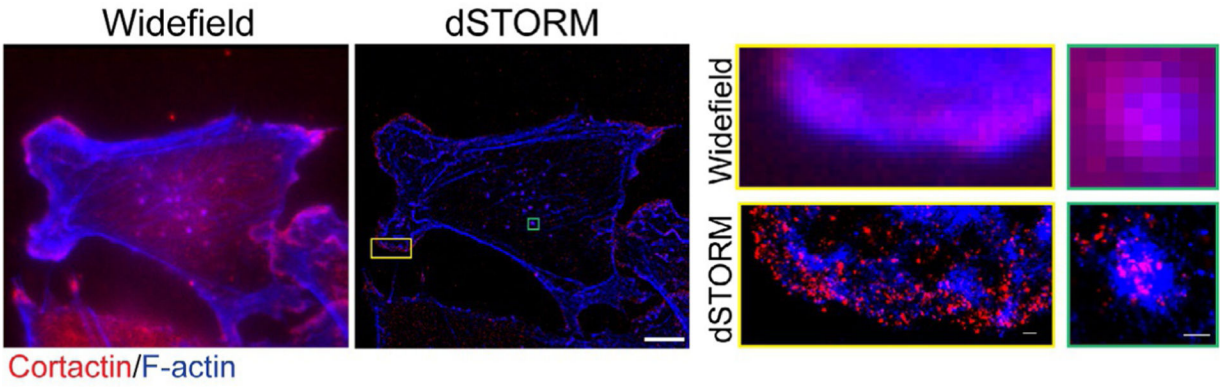

Fig. 5.

dSTORM image of a breast cancer cell. (Left panel): Widefield image of a cancer cell on gelatin matrix. F-actin in blue, cortactin in red. (Middle panel): dSTORM image of the same cancer cell. Top and bottom inset #1 (yellow box): Widefield and dSTORM of a lamellipodia structure. Top and bottom inset #2 (green box): Widefield and dSTORM showing spatial distribution of F-actin and cortactin in an invadopodia structure. Left and middle panel, scale bar: 10μm. Insets, scale bar: 1μm. Data acquisition for dSTORM was carried out on the Nanoimager S (Oxford NanoImaging, ONI, Oxford, UK). Signals from Alexa 647 and Alexa 488 were recorded sequentially for 10,000 frames each. Localization and image rendering were performed in the NimOS v1.4 software, and the final reconstruction displayed in a precision mode.