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SPECIAL ISSUE: 3D CELL BIOLOGY

COMMENTARY

Microtubules in 3D cell motility

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ABSTRACT

Three-dimensional (3D) cell motility underlies essential processes, such as embryonic development, tissue repair and immune surveillance, and is involved in cancer progression. Although the cytoskeleton is a well-studied regulator of cell migration, most of what we know about its functions originates from studies conducted in two-dimensional (2D) cultures. This research established that the microtubule network mediates polarized trafficking and signaling that are crucial for cell shape and movement in 2D. In parallel, developments in light microscopy and 3D cell culture systems progressively allowed to investigate cytoskeletal functions in more physiologically relevant settings. Interestingly, several studies have demonstrated that microtubule involvement in cell morphogenesis and motility can differ in 2D and 3D environments. In this Commentary, we discuss these differences and their relevance for the understanding the role of microtubules in cell migration *in vivo*. We also provide an overview of microtubule functions that were shown to control cell shape and motility in 3D matrices and discuss how they can be investigated further by using physiologically relevant models.

KEY WORDS: +TIP, 3D matrix, Rho GTPase, Cancer, Cell migration, Microtubule

Introduction

Since the early studies in the middle of the 20th century that linked the cytoskeleton and cell migration, a considerable research effort led to identification of actin filaments and microtubules as master regulators of cell shape and motility (Etienne-Manneville, 2013; Le Clainche and Carlier, 2008). It became increasingly clear that the molecular interplay between actin and microtubules ultimately controls cell protrusion and adhesion to the extracellular matrix (ECM). The crosstalk between actin and microtubules is tightly connected to the regulation of the small GTPases Rho, Rac1 and Cdc42, and determines cell polarity, actin polymerization and actomyosin contractility (Rodriguez et al., 2003).

Actin filaments (F-actin) are polar fibers that result from the polymerization of actin monomers under the control of nucleation and elongation factors (Carlier et al., 2015). Because of its capacity to organize into branched networks that can push membranes or contractile structures, such as stress fibers and cortex-associated meshes, F-actin is viewed as the main cytoskeletal component that physically controls cell shape and adhesion in 2D and 3D (Blanchoin et al., 2014; Case and Waterman, 2015; Pollard and Cooper, 2009).

Microtubules are polarized tubes built of α- and β-tubulin heterodimers (Desai and Mitchison, 1997). Their plus ends display frequent growth and shrinkage, and are the binding sites for complexes composed of so-called plus-end-tracking proteins (+TIPs) that control microtubule polymerization, signaling and interaction with cellular structures (Akhmanova and Steinmetz, 2015). In models of 2D cell adhesion and movement, microtubules are mainly considered as regulators of Rho GTPase signaling and transport of adhesion receptors, such as integrins (Etienne-Manneville, 2013). Classic experiments, in which microtubules were disassembled by adding the depolymerizing drug nocodazole, showed that microtubules activate Rac1 and inhibit Rho (Krendel et al., 2002; Waterman-Storer et al., 1999). In 2D, treatment with nocodazole causes cell protrusion defects due to reduced Rac1-driven actin polymerization and increased cell contractility in response to Rho-myosin II signaling (Krendel et al., 2002; Waterman-Storer et al., 1999). Although the molecular mechanisms underlying these effects are not entirely clear, the integrity of the microtubule network is a well-established player in controlling the balance of Rho GTPase activities and, therefore, F-actin assembly and actomyosin contractility in 2D cultures (Etienne-Manneville, 2013). Interestingly, specific cell types, such as glioblastoma cells have been reported to be actin independent but require microtubules for migration in 2D (Panopoulos et al., 2011).

An important process in the regulation of cell migration is the interaction between dynamic microtubules and integrin-based ECM adhesions, i.e. focal adhesions (FAs), that was described in the late 1990s (Kaverina et al., 1998). Specialized cortical complexes that involve +TIPs were shown to modulate this interaction (Lansbergen et al., 2006; van der Vaart et al., 2013; Wu et al., 2008; Bouchet et al., 2016), and the majority of existing data suggest that microtubules promote FA turnover (Stehbens and Wittmann, 2012) (Fig. 1). This function has been linked to Rho inhibition, integrin endocytosis and matrix protease exocytosis, and is thought to be crucial for cell motility on stiff 2D substrates (Stehbens and Wittmann, 2012; Stehbens et al., 2014).

In vivo, processes as diverse as embryonic development, tissue homeostasis, immune surveillance and cancer invasion rely on cell migration through soft 3D matrices, such as connective tissue or basement membranes (Even-Ram and Yamada, 2005). Over the last three decades, biology of the cytoskeleton greatly benefited from the advances in light-microscopy of thick specimens and *ex vivo* 3D-culture systems (Fischer et al., 2011; Shamir and Ewald, 2014). Obviously, studies of cells cultured on stiff 2D substrates paved the way to our understanding of cell migration, but the analysis of 3D models is now needed to uncover the role of microtubules in cell motility in a more-physiological context. In fact, compared to F-actin and its regulators, the contribution of microtubules to 3D cell motility appears underexplored (Petrie and Yamada, 2012; Riching and Keely, 2015). Here, we review the current body of data that establishes a clear link between microtubules and cell motility in 3D by focusing mostly on soft 3D matrices, and discuss similarities and differences with the results from classic 2D models when available. We also address the aspects of microtubule functions that we think are of particular interest for future investigation in 3D cell migration, and that might be relevant for physiological and pathogenic processes.

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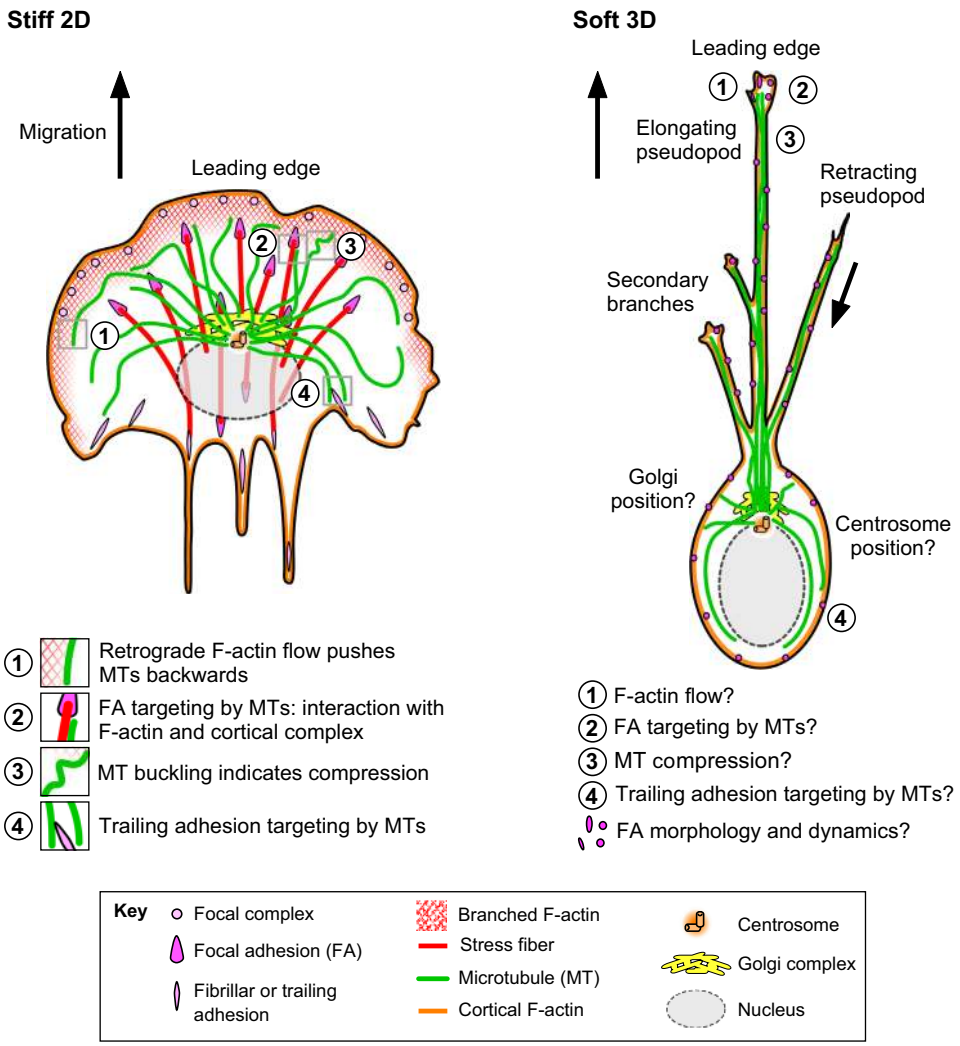

Fig. 1. Microtubule organization in mesenchymal cells on stiff 2D and in soft 3D matrices. (Left) On stiff 2D substrates, mesenchymal cells adopt a polarized morphology with a leading edge that is characterized by a branched F-actin network. F-actin pushes the membrane forward and contributes to focal complex formation. Maturation of focal adhesions (FAs) is associated with formation of stress fibers. At the back of the cell, adhesions are pulled forward during migration (trailing adhesions). Microtubule (MT) minus ends are mainly anchored at the centrosome or the Golgi complex. Microtubule plus ends extend into the lamella and the cell rear where they are in close proximity to the cortex. Microtubules are destabilized by retrograde F-actin flow (1) and stabilized when interacting with microtubule–actin crosslinking proteins (e.g. MACF1) and cortical complexes near FAs (2). Microtubule buckling near the cell edge suggests compression (3). Targeting of FAs by microtubules favors FA disassembly (4). (Right) In soft 3D matrices, lamellae are replaced by pseudopods that show microtubule growth and shrinkage. FA dynamics and morphology, as well as interplay between microtubules, F-actin, FAs and forces are not yet characterized. Moreover, the exact position of the Golgi complex and the centrosome, the mechanisms underlying their positioning and the importance of the precise localization of these structures for 3D migration are still unknown.

Microtubule requirement in cell morphogenesis and motility in 3D

The vast majority of data available about the role of microtubules in 3D cell migration (i.e. invasion) originates from *in vitro* studies and will be the main focus here. Since the publication of the first *in vitro* cell culture system based on collagen hydrogels in the 1970s (Elsdale and Bard, 1972), the development and standardization of well-controlled 3D culture setups combined with advanced live imaging and genetic manipulations grew exponentially (Shamir and Ewald, 2014). Such approaches are now largely considered to be superior to 2D cultures in modeling the behavior of motile cells in most of the physiological contexts. The emergence of these culture systems was concomitant with the seminal descriptions of morphology and behavior in 3D matrices of different types of motile cell including fibroblasts (Bard and Hay, 1975; Bell et al., 1979; Bellows et al., 1981; Elsdale and Bard, 1972), developmental precursors, such as neural crest cells (Davis, 1980), or endocardial cushion cells (Bernanke and Markwald, 1982), leukocytes (Grinnell, 1982) and endothelial cells (Schor et al., 1983).

Cells use different modes of motility to move through 3D matrices, with mesenchymal and amoeboid migration being the two main types (see Box 1). The importance of microtubules for mesenchymal cell morphogenesis in soft 3D matrices was initially demonstrated in fibroblasts grown in collagen gels (Bell et al., 1979; Tomasek and Hay, 1984). Microtubule depolymerization following treatment with colcemid inhibits the bipolar and elongated morphology of fibroblasts in 3D (Elsdale and Bard, 1972), inducing a ‘pear’ shape of the cell (Bell et al., 1979). Later, both microtubule-stabilizing (paclitaxel, i.e. Taxol) and -destabilizing (nocodazole) drugs were shown to induce fibroblasts to lose long protrusions, which they normally form in collagen gels (Tomasek and Hay, 1984). These protrusions, which were associated with 3D cell motility early on (Bard and Hay, 1975; Schor et al., 1980; Schor, 1980), will, hereinafter, be referred to as pseudopods (Fig. 1).

A few years later, the importance of microtubules for cell morphogenesis was reported to strikingly differ in soft collagen gels compared to cultures on 2D plastic dishes (Unemori and Werb, 1986). In this study, consistent with earlier research (Ivanova et al., 1976; Vasiliev et al., 1970), colcemid-based microtubule disassembly did not abolish spreading of fibroblasts on solid 2D supports but affected pseudopod-based cell elongation on soft collagen matrices (Unemori and Werb, 1986). This result was later reproduced by nocodazole-induced microtubule disassembly in cells cultured in soft collagen gels compared to stiff 2D substrates (Rhee et al., 2007). In addition, treatment with nocodazole was shown to abolish fibroblast motility in 3D matrices (Doyle et al., 2009). Importantly, the dependence of pseudopod elongation on microtubules was also observed in cancer cells that display a mesenchymal morphology in 3D, such as MDA-MB-231 cells

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Box 1. Cell morphogenesis and migration in soft 3D matrices

The majority of studies that link microtubule function and 3D cell migration were performed in soft substrates (elastic modulus of a few hundred Pa), such as collagen-I-based gels or Matrigel (Soofi et al., 2009; Wolf et al., 2013). It is clear that some physiological situations provide 1D and 2D environments for cells to migrate: for example, cancer cells that leave the tumor often move between sheets of tissues, e.g. the surface of muscle fibers. However, soft collagen-rich matrices are considered to mimic well multiple types of *in vivo* tissue environments (Doyle et al., 2009; Petrie et al., 2012). Early research using soft collagen gels and *ex vivo* cultures led to the definition of two main 3D motility modes: (i) amoeboid cell migration, as seen in leukocytes (Lam and Huttenlocher, 2013; Lammermann and Germain, 2014) and (ii) mesenchymal cell migration, found in fibroblasts, invasive cancer cells and many developmental precursors, especially during EMT (Friedl and Wolf, 2010; Lim and Thiery, 2012). Amoeboid migration is characteristic for rounded cells with relatively low ECM adhesion and relatively high Rho-driven contractility, whereas mesenchymal migration depends on cell elongation associated with relatively high ECM adhesion and Rac1-driven protrusion (Friedl and Wolf, 2010). The shape of mesenchymal cell protrusions depends on stiffness: on stiff substrates, they form lamellae as opposed to pseudopods formed on soft substrates (see Fig. 1). Other cell migration modes in 3D matrices depend on pressure-based protrusions, such as blebs and lobopodia, but the role of microtubules in this context is poorly understood (Petrie and Yamada, 2016). Importantly, 3D cell migration *in vivo* displays significant plasticity that allows, in certain physiological or pathogenic situations, to switch between collective and single cell behavior (Friedl and Gilmour, 2009; Mayor and Etienne-Manneville, 2016). Of note, several basic principles of single cell motility also apply to collective migration, for example, in the leader cells (Mayor and Etienne-Manneville, 2016).

(Kikuchi and Takahashi, 2008; Oyanagi et al., 2012). High doses of paclitaxel or nocodazole blocked migration of these cells in soft collagen gels (Carey et al., 2015). In fact, even low doses of different microtubule-targeting agents (MTAs), which were insufficient to block cell division, impaired matrix invasion by MDA-MB-231 cells (Tran et al., 2009). Microtubule requirement for pseudopod-based motility in 3D was also tested and confirmed by MTA-based approaches in endothelial cells (Lyle et al., 2012; Martins and Kolega, 2012; Pourroy et al., 2006). Nocodazole treatment also caused defects in elongation of invadopodia, which are small specialized protrusions observed in tumor cells that are thought to play a role in cancer invasion (Di Martino et al., 2016; Schoumacher et al., 2010). The formation of podosomes, which share similarity with invadopodia and are specialized adhesive and invasive structures involved in 3D migration of certain cell types (e.g. macrophages, endothelial cells), also depends on microtubules (Linder et al., 2000; Maridonneau-Parini, 2014; Seano and Primo, 2015). Together, these studies suggest a universal role for microtubules in mesenchymal cell protrusion and elongation within soft environments.

Pseudopod loss induced by microtubule disassembly strongly suppresses cell motility in soft 3D matrices but has a much milder effect on cell movement on top of soft 2D matrices (Myers et al., 2011). The specific requirement of long pseudopods for mesenchymal motility that occurs inside soft 3D matrices and not on top of soft 2D matrices is most probably due to the fact that 3D migration depends on distant adhesions, which would provide anchoring points for contraction-based cell body displacement (Friedl and Alexander, 2011). In 2D, even in the absence of elongated pseudopods and provided that adhesion is maintained, mesenchymal cells are expected to move freely at the surface of the matrix because they are not limited by the meshwork pore properties (Wolf et al., 2013).

In parallel to this work, data on the requirement of microtubules for amoeboid migration in 3D (Box 1) has also been collected (Lam and Huttenlocher, 2013). A collagen-based 3D culture of leukocytes was developed in the 1980s (Grinnell, 1982). Later studies that have used this system established that microtubules have a limited role in amoeboid migration in 3D because various MTAs did not abrogate leukocyte motility in soft collagen gels (Nikolai et al., 1999; Ratner et al., 1997). This idea was strengthened by *in vivo* studies in zebrafish, which demonstrated that 3D migration of leukocytes, such as macrophages (Redd et al., 2006) and neutrophils (Yoo et al., 2012), is not abolished by microtubule depolymerization. However, microtubules are required for the directionality of amoeboid cell migration in 3D (Redd et al., 2006; Yoo et al., 2012). The uropod, a specialized structure formed at the rear of cells that migrate by using an amoeboid motility mode, is important for directionality of movement (Hind et al., 2016). Interestingly, also in 3D, microtubules populate this protrusion as observed in leukocytes migrating in soft collagen gels (Ratner et al., 1997) as well as in *in vivo* (Yoo et al., 2012), and are thought to regulate actin assembly and actomyosin contractility (Hind et al., 2016). Notice that the term 'amoeboid migration' covers a spectrum of motility modes driven by cytoskeletal mechanisms that can vary (Lammermann and Sixt, 2009) and might thus have different microtubule requirements.

Both single cell and collective migration during development are spectacular examples of physiological cell motility in 3D (Aman and Piotrowski, 2010; Keller, 2005; Kurosaka and Kashina, 2008). Microtubule organization has been the subject of early electron microscopy studies in migrating myogenic cells (Jacob et al., 1978), epiblasts that acquire invasive properties due to epithelial-mesenchymal transition (EMT) during gastrulation (Granholm and Baker, 1970) and cardiac cushion cells grown in collagen gels (Bernanke and Markwald, 1982). A recent example of the effect of microtubule depolymerization on developmental processes is a study of protrusion formation during epithelial tissue sealing (Eltsov et al., 2015).

3D cell migration is promoted by EMT and is characteristic for mesoderm progenitors during gastrulation and movement of neural crest cells (Aman and Piotrowski, 2010). Although microtubule disassembly in response to nocodazole causes premature breakdown of the basement membrane during gastrulation (Nakaya et al., 2008), microtubule functions during mesoblast migration in 3D are poorly understood. Studies that used paclitaxel and nocodazole on migrating melanoblasts – cells that originate from neural crest cells – provided an elegant *in vivo* example of the role microtubules have in developmental cell motility in 3D (Li et al., 2011a; Thomas and Erickson, 2008). Similarly to other mesenchymal cells in 3D cultures, drug-based microtubule network disorganization caused mouse melanoblasts to lose their long pseudopods and stop migrating (Li et al., 2011a). For neural crest cells, the correlation between pseudopod loss and motility impairment appears to be specific for the 3D situation as nocodazole-treated neural crest cells in 2D cultures still move, although they round up (Moore et al., 2013). Finally, neuron migration within a 3D environment is crucial for brain development, and 3D systems are used to study this process (Gil and del Rio, 2012). It is now firmly established that both microtubule organization and microtubule-based motors, such as dynein and its cofactors, are crucial for neuronal migration, and their inactivation can cause brain development defects. This large body

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of research is covered by excellent reviews and is, therefore, not further discussed here (Coles and Bradke, 2015; Kapitein and Hoogenraad, 2015; Moon and Wynshaw-Boris, 2013).

Microtubule dynamics and functions have been extensively explored in 2D systems, and now need to be systematically tested in relevant 3D models. The dramatic difference of microtubule requirement in cell morphogenesis within stiff and soft matrices (Rhee et al., 2007; Unemori and Werb, 1986) suggests that what we have learnt by using 2D cultures does not necessarily apply to physiological environments – typically 3D *in vivo* – and, thus, should be revisited. This is already happening and, in the following, we review mechanisms that directly link microtubules and cell motility in 3D environments.

ECM adhesion and trafficking in 3D environments

One of the most-studied functions associated with microtubules in 2D cell migration is the vesicular transport of adhesion receptors (Etienne-Manneville, 2013; Stehbens and Wittmann, 2012). More specifically, integrin recycling via endocytic pathways has been extensively explored and shown to regulate FA dynamics, which is essential for cell motility in 2D cultures (Paul et al., 2015; Schiefermeier et al., 2011) (Fig. 2). Mechanistically, integrin recycling is one of the clearest links between microtubules and cell motility in 3D matrices (Jacquemet et al., 2013; Paul et al., 2015). Yet, compared to 2D migration, microtubule-dependent regulatory mechanisms linked to trafficking in 3D migration are still underexplored. A well-documented pathway that controls cancer cell migration in 3D is the recycling of α5β1 integrin. This is regulated by Rab25, a protein related to Rab11 that localizes to recycling and late endosomes, and by the chloride intracellular channel 3 (CLIC3), a protein of late endosomes and lysosomes (Caswell et al., 2007; Christoforides et al., 2012). Another example is the Rab4-dependent recycling of αvβ3 integrin, which promotes the invasion of cancer cells in 3D matrices with low fibronectin composition (Christoforides et al., 2012).

Although a link exists between integrin recycling and the ability of a cell to move in 3D matrices by using ECM adhesion and long pseudopods, the difficulties in performing 3D microscopy have been a strong impediment for detailed analysis of this process. Few examples of alternative culture methods have been developed thus far in order to overcome the limited working distance of the optics that are available for live fluorescence imaging. These include cell-derived matrices that are secretion products of high-density cell cultures; they allow formation of fibrillar 2D cultures, which were used to image the endosomal markers Rab25 and Rab11 in cancer cell pseudopods (Caswell et al., 2007; Dozynkiewicz et al., 2012; Jacquemet et al., 2013). Furthermore, vesicle-associated membrane protein 3 (VAMP3) was imaged in pseudopods formed by canine MDCK cells that had been stimulated with hepatocyte growth factor (HGF) and grown in hybrid Matrigel/collagen I cultures (Gierke and Wittmann, 2012). However, the connection between this endosomal protein and 3D cell migration still needs to be clarified (Kean et al., 2009).

Caveolae represent another type of membrane structure that is thought to contribute to integrin recycling, FA turnover and directional cell migration (Echarri and Del Pozo, 2006; Grande-Garcia and Del Pozo, 2008; Stehbens and Wittmann, 2012). In 2D, microtubule plus-end destabilization has been associated with increased trafficking of caveolae components and their reduced delivery to the plasma membrane (Wickstrom et al., 2010). The

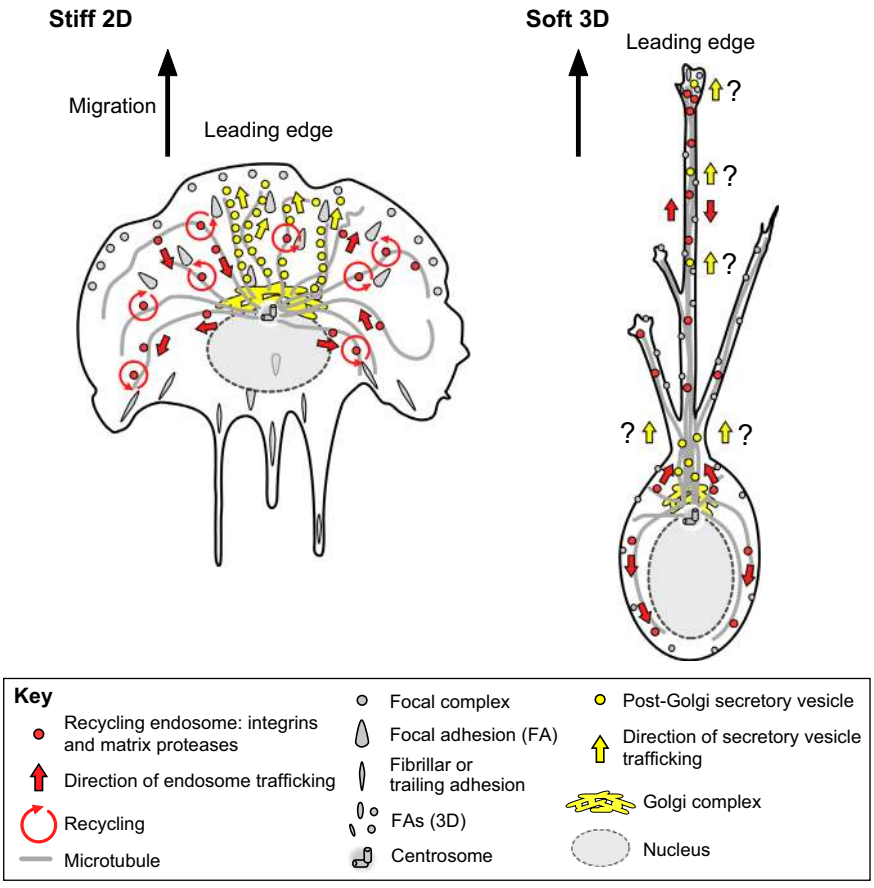

Fig. 2. Organization of trafficking in mesenchymal cells on stiff 2D and soft 3D matrices. (Left) On stiff 2D substrates, recycling endosomes are involved in the delivery of integrins and matrix proteases to the membrane. Endosomes involved in integrin trafficking undergo both short- and long-distance transport. Post-Golgi carriers display polarized trafficking towards the leading edge where exocytosis occurs, notably near FAs. (Right) In soft 3D matrices, endosomal transport is also multi-directional but post-Golgi secretory trafficking has not yet been characterized.

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expression of the caveolae-associated protein caveolin-1 has been linked to 3D matrix invasion in cancer cells (Hayashi et al., 2001; Lin et al., 2005) and endothelial cells (Parat et al., 2003). However, the exact functions of caveolae in 3D cell motility and how they relate to microtubules remain to be elucidated.

In 2D migrating cells, post-Golgi secretory vesicles were shown to be targeted in a polarized way to the leading cell edge, where they undergo exocytosis (Schmoranzer et al., 2003; Schmoranzer and Simon, 2003) (Fig. 2). This function regulates cell motility on 2D substrates and depends on the organization of the Golgi complex through the +TIPs cytoplasmic linker associated protein 1 and 2 (CLASP1 and CLASP2, respectively) (Miller et al., 2009; Yadav et al., 2009), although the contribution of the post-Golgi carriers to ECM adhesion in 3D matrices remains to be explored.

2D culture studies also demonstrated that vesicles carrying matrix proteases are targeted to FAs and might contribute to motility by disrupting integrin engagement with the ECM (Stehbens et al., 2014; Takino et al., 2007; Wang and McNiven, 2012). The matrix metalloproteinase 14 (MMP14, hereafter referred to as MT1-MMP) is required for endothelial cell migration in 3D (Genis et al., 2006), and involved in several developmental and pathogenic processes that rely on 3D matrix invasion (Bonnans et al., 2014). Moreover, MT1-MMP in conjunction with the physical properties of ECM is a determining factor in mesenchymal cell migration in 3D (Wolf et al., 2013). MT1-MMP trafficking and delivery to the plasma membrane are microtubule dependent, involve late endosomes and are required for mesenchymal invasion by cancer cells (Frittoli et al., 2014; Macpherson et al., 2014; Remacle et al., 2005; Rosse et al., 2014). It should be noted that delivery of MT1-MMP to the plasma membrane is also controlled by F-actin and its regulator N-WASP (Yu et al., 2012), and the respective contributions of microtubule- and actin-based mechanisms of MT1-MMP trafficking in 3D will thus have to be further investigated.

Microtubule-related signaling in 3D environments

Similar to adhesion receptor trafficking, most of the data available on signaling downstream of microtubules originate from 2D studies. Microtubule-dependent signaling includes regulation of Rho GTPases that locally control actin polymerization, actomyosin contractility and FA assembly (Etienne-Manneville, 2013). The demonstration that microtubule depolymerization (e.g. in response to nocodazole treatment) downregulates Rac1 has stimulated the attempts to identify the Rho GTPase regulators that can link microtubules to cell protrusion and migration (Fig. 3). The Rac1 activator ARHGEF4 (hereafter referred to as Asef) binds mutants of tumor suppressor adenomatous polyposis coli protein (APC) – a tumor suppressor and +TIP – that are associated with colon cancer, and this interaction is thought to activate Asef (Kawasaki et al., 2000, 2003). Because Rac1 promotes actin polymerization and lamellipodium extension, this mechanism was proposed to be linked to a pro-migratory function of microtubules in pathogenic situations, although this had only been tested in 2D cultures. Another Rac GEF, TIAM2 (also known as STEF) was shown to be required for FA disassembly during nocodazole washout in 2D cultures (Rooney et al., 2010), suggesting that TIAM2 is activated by re-growing microtubules. Other Rac1 activators, including the +TIP triple functional domain protein (TRIO), which is a Rac1 and RhoG GEF (Blangy et al., 2000; van Haren et al., 2014), as well as TIAM1 – which interacts with microtubule-associated protein 1B (MAP1B) and is a Rac1 GEF (Montenegro-Venegas et al., 2010) – might also participate in microtubule-dependent cell protrusion and migration.

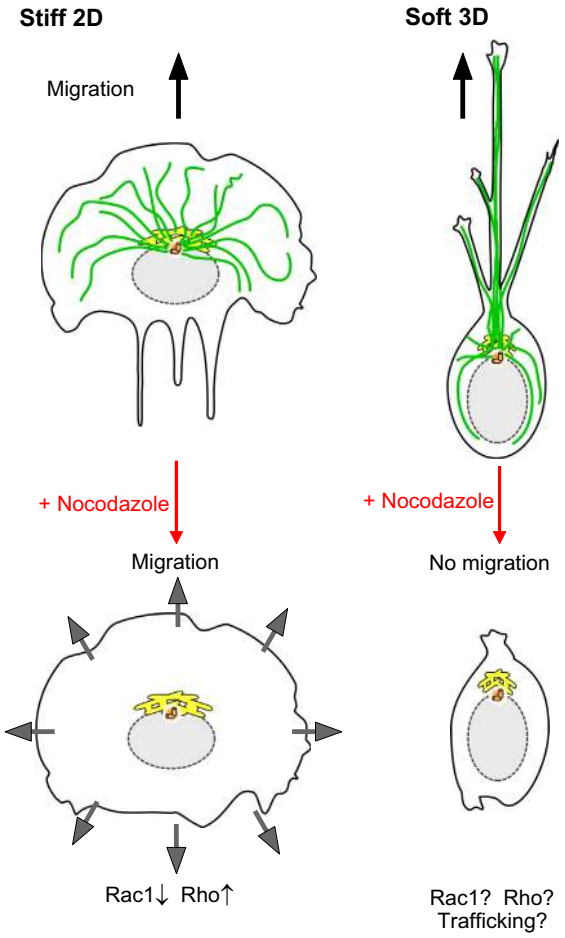

Fig. 3. Effects of microtubule disassembly in mesenchymal cells grown on stiff 2D and in soft 3D matrices. (Left) On stiff 2D substrates, microtubule disassembly (e.g. in response to addition of nocodazole) does not abolish cell spreading but reduces Rac1 activity, lamellipodium protrusion and the directionality of the cell, but increases Rho activity. (Right) In soft 3D matrices, microtubule disassembly in response to nocodazole causes cell rounding and blocks mesenchymal cell migration. Here, the effects on Rho, Rac1 and membrane trafficking are still unclear.

Microtubules have also been linked to the inhibition of Rho and, therefore, contractility driven by myosin II (Fig. 3). GEF-H1 (also known as ARHGEF2) is thought to be inactive when bound to microtubules; it is activated upon microtubule depolymerization, leading to Rho activation and cell contraction (Chang et al., 2008; Krendel et al., 2002). GEF-H1 was also proposed to promote Rho activation at the leading edge and locally favor actin polymerization and lamellipodium protrusion in 2D (Nalbant et al., 2009). However, GEF-H1 action might be more complicated because it is possibly also a Rac1 GEF (Callow et al., 2005; Ren et al., 1998) and involved in exocytosis – a process that was mentioned above as a putative cell motility regulator (Pathak et al., 2012). Moreover, feedback mechanisms have been described whereby Rac1 stabilizes microtubules by inhibiting the microtubule-destabilizing protein stathmin (STMN1) (Steinmetz, 2007) through p21-activated kinase 1 (PAK1) (Wittmann et al., 2003, 2004). Similarly, Rho has been proposed to stabilize microtubules through the formin mDia1 (also known as DIAPH1); this is independent of actin-nucleating activity of mDia1 but involves binding to the +TIPs EB1 (officially known as MAPRE1) and APC in the lamella of cells grown in 2D (Bartolini et al., 2008; Palazzo et al., 2001; Wen et al., 2004). Furthermore, a

recent study has suggested that mDia1 also forms a complex with another +TIP, CLIP-170 (also known as CLIP1), which acts as a seed for F-actin assembly at microtubule plus ends (Henty-Ridilla et al., 2016). This mechanism will have to be assessed as a new function for microtubules in cell migration involving 3D systems. Interestingly, inhibition of TRIO by Par3 and subsequent Rac1 inactivation has recently been proposed to perturb microtubule growth at cell–cell contacts of migrating neural crest cells *in vivo* (Moore et al., 2013). The proposed model suggests that TRIO and Rac1 drive microtubule stabilization and promote mesenchymal cell protrusion, and that Par3 inhibits this pathway, thereby contributing to contact inhibition of locomotion (Moore et al., 2013). Thus, the functions of TRIO and Rac1 should be further explored in 3D migration models.

The finding that microtubule depolymerization does not abolish fibroblast spreading on stiff substrates, but causes a loss of pseudopods and cell elongation in soft substrates might have implications for microtubule-dependent regulation of Rho GTPases in 3D (Rhee et al., 2007; Unemori and Werb, 1986) (Fig. 3). Inhibition of Rho effectors involved in contractility activation, such as myosin II or its activating kinase Rho-associated protein kinase (ROCK), could not prevent rounding up of fibroblasts in nocodazole-treated soft 3D cultures (Grinnell et al., 2003; Rhee et al., 2007). These results indicate that upregulated Rho activity and contractility are not involved in pseudopod loss upon microtubule depolymerization. An interesting possibility is that Rac1 inactivation and therefore reduced actin-mediated protrusion might explain this effect (Rhee et al., 2007), although this was not yet tested.

A promising approach to assess Rho GTPase regulation in 3D cell migration is the use of biosensors, such as Förster resonance energy transfer (FRET)-based probes (Donnelly et al., 2014). This is exemplified by activity measurements of various Rho GTPases, including Rac1 and Rho, in mesenchymal cancer cells *in vivo* (Hirata et al., 2012; Timpson et al., 2011), in 3D cultures (Hirata et al., 2012; Petrie et al., 2012) and during amoeboid migration of germ cells in zebrafish development (Kardash et al., 2010). Future experiments combining specific manipulation of microtubule dynamics and functions with high-resolution FRET biosensor imaging will help to revisit the link between Rho GTPase regulation and microtubules in 3D systems.

Microtubule-associated proteins in 3D cell migration – specific players

Microtubule functions related to 3D motility are likely to depend on specific microtubule-associated proteins. An interesting example can be found in migrating macrophages of *Drosophila* embryos. Expression of the microtubule-severing protein Spastin impaired the dispersal of these migrating macrophages along the ventral midline of the embryo (Stramer et al., 2010). Mutation of Orbit, the *Drosophila* homolog of the +TIPs CLASP1 and CLASP2, had the same effect and was associated specifically with the alteration of contact inhibition of locomotion. Stramer et al. suggested that the *Drosophila* CLASP promotes formation of a microtubule bundle or 'arm' that is directed towards the leading edge, and that depolymerization of this arm induces rapid cell repolarization upon cell–cell collision and a change in cell trajectory. Because CLASPs suppress microtubule catastrophes, promote microtubule rescue (Akhmanova and Steinmetz, 2015) and are locally enriched at the cortex where they regulate FA turnover (Stehbens et al., 2014), it will be interesting to study their function further during cell migration in a 3D environment.

Other examples that involve +TIPs in cell migration *in vivo* include spectraplakins, which can bind both microtubules and actin (Suozzi et al., 2012). The *Caenorhabditis elegans* spectraplakin VAB-10 is required for the migration of leader cells during gonad development (Kim et al., 2011), and the mammalian MACF1 (also known as ACF7) is necessary for epidermal cell migration *in vivo* (Yue et al., 2016). ACF7 was shown to promote microtubule targeting of FAs by facilitating crosslinking between microtubules and actin (Wu et al., 2008), a function that needs to be further explored within 3D migration.

EB1 is a master regulator of the assembly of +TIP complexes (Akhmanova and Steinmetz, 2015). Although EB1 depletion had a negligible effect on 3D matrix invasion in the mesenchymal-like cancer cells MDA-MB-231 (Morimura and Takahashi, 2011), it reduced pseudopod elongation in HGF-stimulated MDCK cells in 3D cultures (Gierke and Wittmann, 2012). In the latter study, EB1 depletion was associated with diminished phosphorylation of myosin II, a defect in microtubule penetration into pseudopods, and impaired FA formation and vesicle trafficking. EB1 controls the recruitment of numerous +TIPs that are involved in different cellular functions to microtubules, and it will thus be interesting to investigate which partners of EB1 are responsible for the phenotype of EB1 depletion.

Another specific factor that controls microtubule assembly and has been linked to 3D cell motility is the tubulin-binding protein stathmin, a tubulin-sequestering and microtubule-destabilizing protein (Gupta et al., 2013; Steinmetz, 2007). Its inhibition through apoptosis regulatory protein (Siva1) has been associated with microtubule stabilization, reduced 3D matrix invasion, and suppression of EMT and metastasis in a breast cancer model (Li et al., 2011b). Of note, the specific 3D cell migration modalities promoted by stathmin were not examined in this study. Interestingly, the tubulin-sequestering activity of stathmin was shown to promote a change in the morphology of cancer cells grown in soft 3D matrix, with a switch from mesenchymal to amoeboid morphology and migration in 3D (Belletti et al., 2008). Furthermore, cells displaying stathmin-induced amoeboid motility in 3D were more metastatic in mice, suggesting that this morphology switch increases 3D motility *in vivo* (Belletti et al., 2008). This is consistent with the idea that, during 3D matrix invasion, amoeboid migration can be much more efficient than mesenchymal migration (Friedl and Wolf, 2010). Another study has also shown that amoeboid cell migration induced by tumorigenic alterations is associated with microtubule destabilization and increased invasion in 3D (Hager et al., 2012). *In vivo*, the velocity of cells displaying amoeboid motility was increased by nocodazole-induced microtubule destabilization (Yoo et al., 2012). These data suggest that microtubule destabilization by stathmin – or other microtubule-associated proteins yet to be identified – has a pro-migratory effect when amoeboid migration is favored (Friedl and Wolf, 2010; Lammermann and Sixt, 2009; Liu et al., 2015).

Microtubule-severing enzymes such as katanin have been directly linked to the regulation of cell migration in various 2D models (Sharp and Ross, 2012). Furthermore, a recent study has shown that inhibition of the microtubule-severing protein fidgetin-like protein 2 (FIGNL2) promotes epidermal cell migration *in vivo* (Charafeddine et al., 2015), suggesting that the members of this protein family have important roles in 3D cell migration.

In addition to specific microtubule-associated proteins, post-translational modifications of tubulin are emerging as a significant mechanism that controls various microtubule functions, such as the recruitment of +TIPs and microtubule motors (Akhmanova and

Steinmetz, 2008; Etienne-Manneville, 2013; Janke and Bulinski, 2011; Song and Brady, 2015). An interesting example is tubulin acetylation, a modification that is introduced by α-tubulin N-acetyltransferase 1 (ATAT1, hereafter referred to as αTAT1) and is removed by histone deacetylase 6 (HDAC6) (Song and Brady, 2015). In 2D cultured cells, HDAC6 inhibition increases the size of FAs (Bouchet et al., 2011; Tran et al., 2007) and reduces their turnover (Tran et al., 2007). HDAC6 binds the FA protein paxillin that, in turn, inhibits deacetylation (Deakin and Turner, 2014). αTAT1 associates with clathrin-coated pits and FAs at the ventral side of cells grown in 2D culture, and the contact with clathrin-coated pits promotes microtubule acetylation (Montagnac et al., 2013). In 3D environments, microtubule acetylation can regulate transport of the matrix metalloproteinase MT1-MMP; however, its effect on 3D migration appears to be complex because inactivation of both HDAC6 (Rey et al., 2011) and αTAT1 (Castro-Castro et al., 2012; Montagnac et al., 2013) can inhibit 3D matrix invasion.

Multiple microtubule-associated factors, including kinesins KIF1C, KIF5B, KIF3A/B and KIF9, +TIPs EB1 and CLASP1, as well as deacetylase HDAC6, have been linked to the formation of podosomes (Bhuwania et al., 2014; Biosse et al., 2014; Cornfine et al., 2011; Destaing et al., 2005; Efimova et al., 2014; Maridonneau-Parini, 2014; Wiesner et al., 2010; Zhu et al., 2016). Podosomes, which are characterized by specialized organization of actin and adhesion molecules and the ability to degrade ECM, thus appear to depend on different aspects of microtubule regulation and microtubule-based transport for their function.

Microtubules in cell mechanics in a 3D matrix

An often overlooked aspect of microtubule function in cell morphogenesis and migration in 3D matrices is their direct involvement in cell mechanics. A mechanical role for microtubules in cell shape was suggested in early studies (Brown et al., 1996; Dennerll et al., 1988; Janmey et al., 1991; Rudolph and Woodward, 1978; Tomasek and Hay, 1984). In parallel, a link between microtubules and 3D matrix contraction by invasive mesenchymal cells was described in studies that used collagen gels (Bell et al., 1979; Kolodney and Wysolmerski, 1992; Kraning-Rush et al., 2011). Microtubules are required to generate wide-range traction forces in adhesive and elongated cells, such as mesenchymal cells cultured in 3D matrices (Kraning-Rush et al., 2011). This might not only reflect the impact of microtubules on the regulation of adhesion and contractility, but also point to a mechanical function. Early studies suggested that the effect of microtubules on 3D matrix remodeling relates to cell contractility (Danowski, 1989), and this idea was later supported by the link between microtubules and the activation of Rho and myosin II (as discussed earlier). At that time, a more-direct mechanical function of microtubules in regulating the cell shape was, nonetheless, considered (Danowski, 1989). Currently, the idea that a microtubule-dependent control of the balance between Rho and Rac1 is the main contributor to cell elongation in soft 3D matrices is generally favored over a direct mechanical function (Rhee et al., 2007). Yet, compared to F-actin, microtubules are relatively stiff polymers (Hawkins et al., 2010; Mizushima-Sugano et al., 1983) that, during polymerization, can generate pushing forces in the range of a few pN (Dogterom and Yurke, 1997). Therefore, microtubules can push membranes and resist compression (Elbaum et al., 1996; Fygenson et al., 1997a,b; Hotani and Miyamoto, 1990; Waterman-Storer et al., 1995). In fact, the buckling of microtubules in living cells demonstrates that they act as load-bearing fibers (Brangwynne et al., 2006; Robison et al., 2016; Wang et al., 2001). Because of these properties, a mechanical role for microtubules in cell tensegrity (see Box 2) deserves to be further investigated. Interestingly, some recent studies suggest that 3D matrix stiffness itself influences microtubule stability and downstream signaling, such as Rho regulation, but the mechanisms involved are still unclear (Heck et al., 2012; Myers et al., 2011).

A possible limitation of the tensegrity model is that microtubule tips in close proximity to the cortex are mostly dynamic, and actin flow can contribute to their destabilization (Gupton et al., 2002; Waterman-Storer and Salmon, 1997). Moreover, microtubule plus ends are sensitive to compression-induced catastrophes (Janson et al., 2003; Laan et al., 2008), limiting microtubule load-bearing capacity. It is, however, possible that the retrograde actin flow is less in pseudopods of 3D-cultured cells compared to lamellae of 2D-cultured cells, or that specific regulators modulate the sensitivity of microtubule plus ends to depolymerization at locations where a load-bearing microtubule function contributes to cell elongation in 3D (e.g. pseudopod tips in elongated mesenchymal cells) (Fig. 4). It is noteworthy that the movement of cancer cells cultured in strong confinement (i.e. in 3-µm-wide channels) is much more profoundly affected by the inhibition of microtubule polymerization than by that of actin polymerization, actomyosin contractility or integrin adhesion (Balzer et al., 2012). Here, confinement redirected microtubule polymerization towards the leading edge, suggesting that growing microtubules provide force for advancement of the cell edge (Balzer et al., 2012). These data support the idea that, in certain 3D situations, microtubule–cortex interactions are regulated in a way that promotes their mechanical role during cell protrusion.

Box 2. Microtubules and cell tensegrity in soft 3D matrices

The cell morphogenesis model called ‘tensegrity’ proposes that microtubules function as compression struts to modulate cell shape (Ingber, 2003) (see Fig. 4). In this model, cell shape in a soft matrix depends on continuous membrane tension and is greatly influenced by the ability of microtubules to resist compression. Briefly, pre-stress that is generated by myosin-II-based contractility and/or isometric membrane tension promotes cell rounding, especially when the matrix is compliant and/or ECM adhesions are dislodged. In other words, in soft and/or poorly adhesive substrates, more compression forces are transferred to the cytoskeleton. Mesenchymal cells grown in soft collagen-based gels correspond to such a situation, as FA formation is limited in these cells compared to 2D cultures on stiff supports (Harunaga and Yamada, 2011; Rhee et al., 2007). Accordingly, the dependence of a cell on microtubules for morphogenesis and particularly elongation might be higher. Two results from a study by Rhee et al. potentially support a direct mechanical role of microtubules in cell elongation in soft matrices (Rhee et al., 2007). First, in fibroblasts grown on highly adhesive and stiff 2D matrices, spreading does not depend on microtubules but long cell protrusions become highly microtubule-dependent when myosin II is inhibited – a treatment that typically dislodges ECM adhesion (Burridge and Guilluy, 2016). Second, in soft 3D matrices, where ECM adhesion becomes limited, long protrusions become fully dependent on microtubules, with or without myosin II activity. These data indicate that, in situations where ECM adhesion is reduced, fibroblast elongation becomes more dependent on microtubules. Therefore both the altered signaling (e.g. reduced Rac1 activity) and impaired resistance to compression could contribute to the rounding up of mesenchymal cells in 3D upon treatment with nocodazole (Fig. 4).

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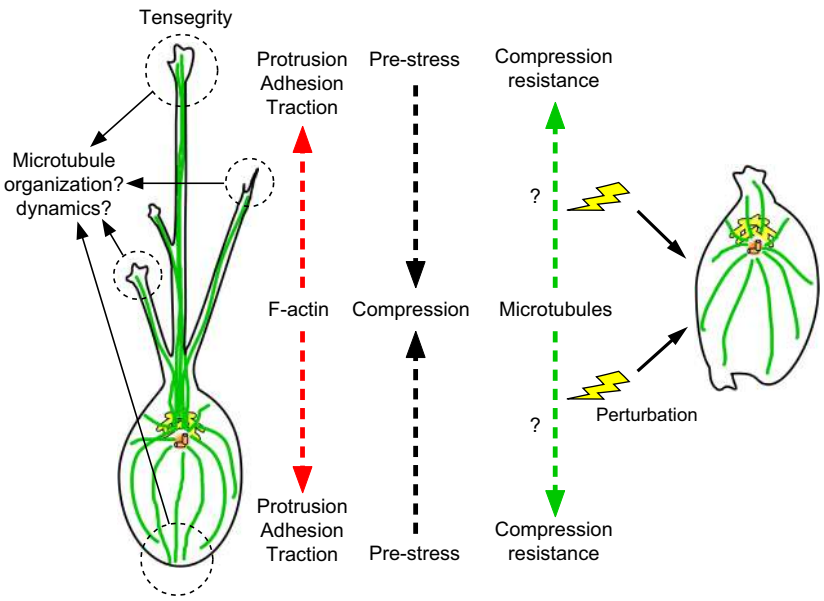

Fig. 4. Potential role of microtubules in cell tensegrity in soft 3D matrix. In mesenchymal cells grown within a 3D matrix, F-actin is the main driver of protrusive and adhesion-based traction forces. Cellular pre-stress generated by several factors (e.g. contractility) promotes compression of the cytoskeleton. Microtubules can potentially mediate compression resistance during mesenchymal cell elongation in 3D matrices. Perturbation of either mechanical or signalling-based functions in compression resistance induces cells to round up. To better understand the role of microtubules in soft-matrix-based tensegrity, an important question is how microtubules are organized and controlled at elongating and retracting pseudopod tips and at the cell rear.

Microtubule alteration and diseases related to cell migration

Some of the microtubule alterations observed in human disease were found to be associated with pathogenic cell migration *in vivo*. In addition, misregulated gene expression and mutations that, potentially, cause microtubule defects have been described in metastatic cancers. These include aberrant expression of conventional microtubule-associated proteins, and mutation or altered expression levels of tubulin isoforms (Kavallaris, 2010). For example, stathmin overexpression is associated with poor prognosis in metastatic cancers (Belletti and Baldassarre, 2011). Inactivation of the tumor suppressors p53, $p21^{Cip1}$ and $p27^{Kip1}$, which is frequently found in cancers, has been associated with alterations in microtubule dynamics (Baldassarre et al., 2005; Bouchet et al., 2011; Galmarini et al., 2003). The expression or function of other microtubule regulators, such as survivin (Chen et al., 2016; Rosa et al., 2006), ATIP3 (Molina et al., 2013; Velot et al., 2015) and the +TIPs EB1 (Liu et al., 2009; Stypula-Cyrus et al., 2014) and APC (Etienne-Manneville, 2009), was found to be altered in certain cancers, but the exact role of these proteins in 3D cell migration is still unclear. Mutations in genes encoding tubulin isoforms and different microtubule regulators including +TIPs have also been associated with neurodevelopmental diseases, including disorders of neuronal migration (Breuss and Keays, 2014; Chakraborti et al., 2016; van de Willige et al., 2016), and it will be interesting to investigate which of their functions reflect general mechanisms of 3D cell motility and which are neuron specific.

Microtubule perturbation not only represents a cause of human disease but also an important therapeutic strategy. MTAs have been at the forefront of anti-metastatic therapies for decades, and their discovery is still a very active field of research (Dumontet and Jordan, 2010). Although MTAs are mostly regarded to be mitosis-blocking agents, there is increasing evidence that interphase cells and, thus, migrating cancer cells, represent their major target *in vivo* (Janssen et al., 2013; Komlodi-Pasztor et al., 2011; Mitchison, 2012). Inhibition of cell migration emerges, therefore, as a promising direction for the therapy of metastatic cancer (Cheung and Ewald, 2014; Palmer et al., 2011) with the aim to not only target tumors but also invasion of endothelial cells in tumors. To identify the molecular mechanisms that control microtubule function in 3D cell motility is, thus, of major importance.

Concluding remarks

For several decades, cytoskeleton studies aimed to address microtubule functions have focused on cells cultured on stiff 2D substrates. This research defined microtubules as an essential scaffold for polarized trafficking and signaling, and provided tools to investigate microtubule functions in now easily accessible cell culture models by using soft 3D matrices. This, in many cases, provides a better mimic of physiological tissue environments. The assessment of adhesion and actin regulators in 3D models has already generated considerable advances in our comprehension of cytoskeletal functions within the context of 3D cell motility and the great plasticity of this process observed *in vivo* (Paul et al., 2015; Petrie and Yamada, 2016). The exciting new field of mechanobiology integrates matrix properties with cytoskeletal dynamics and addresses how the feedback relationships between them fine-tune cell behavior in 3D environments (Charras and Sahai, 2014). The role of microtubules in these processes now needs to be carefully evaluated. Characterization of specialized microtubule-associated proteins with various functions, i.e. +TIPs and their minus-end-associated counterparts (−TIPs) (Akhmanova and Hoogenraad, 2015), provides opportunities to test microtubule functions in 3D cell migration in a more specific way. Finally, the development of light-sheet microscopy and its recent application to microtubule plus-end dynamics in 3D (Chen et al., 2014; Wu et al., 2013; Yamashita et al., 2015) combined with the development of more sophisticated 3D tissue models, such as organoids (Shamir and Ewald, 2014), or with microfabricated and microfluidic devices (Paul et al., 2016), has initiated a new era of cytoskeletal studies in live cells that migrate in 3D environments. Important questions for future research include how microtubule–actin interactions, microtubule-related signaling, adhesion regulation and trafficking, and mechanical properties within soft 3D matrices compare to what is known from decades of 2D biology. Both *in vivo* models and 3D culture systems of defined chemical and biophysical properties combined with high-resolution microscopy should provide new possibilities to tackle these issues and will, in the coming decades, surely reveal exciting knowledge about microtubule functions.

Competing interests

The authors declare no competing or financial interests.

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