Advanced microscopy studies of invadosome rosettes

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Science is not perfect, it is often misused. It's only a tool, but it is the best tool we have. Self correcting, ever changing, applicable to everything.

Carl Sagan

7 | Summarizing discussion

Invadosomes among other cellular protrusions

To interact with and to move through the extracellular environment, eukariotic cells display a whole range of actin-based structures at their peripheries. This versatile group comprises lamellipodia, filopodia, lobopodia, blebs, peripheral ruffles, circular dorsal ruffles, actin waves and finally invadosomes. Invadosomes themselves come in different shapes and flavors, from podosomes, relatively big (0.5 – 1 μm) and constantly palpating their environment, invadopodia, more slender and protrusive and invadosomes in Src-transformed cells to peripheral belts and dynamic rosettes the signaling (**Chapter 3**) and dynamics (**Chapter 5**) of which are the subject of this thesis. This wealth of forms can be displayed due to the great flexibility of the actin cytoskeleton. Constantly rearranged and bundled nanometer-thick actin fibers are the subject of complex regulation that not only enables their remodeling into columns, veils or rods at a micrometer scale but also facilitates their orchestrated movement at even larger scales.

In **Chapter 2** we reviewed the current understanding of actin networks within invadosomes. Although column-like invadosomes more closely resemble the protrusive filopodia than the veil-like lamellipodia, invadosomes contain both bundled actin fibers, characteristic of filopodia and branched actin networks characteristic of lamellipodia. It is therefore only natural that invadosomes also share many characteristic actin organizing proteins with both of the above structures. Although a lot is known about the actin arrangement and regulation within invadosomes themselves, our understanding of these processes is far from complete and new studies are constantly adding different players to the emerging picture. For example, during preparation of this thesis, two reports were published that describe new actin bundlers regulating structure and function of invadosomes. Hoffmann and colleagues identified cystein-rich protein 2 (CRP2) to be responsible for formation of thick actin bundles in extended actin cores of invadopodia of invasive breast cancer cells [449]. They reported CRP2 to be specific for the invasive structures as the knockdown of this protein targeted the invasive potential of the cells but did not alter their 2D migration patterns [449]. Furthermore, Van Audenhove and colleagues showed that the known actin bundler fascin is accompanied in invadopodia by another bundler, L-plastin [450]. Interestingly, fascin and L-plastin cooperate but cannot compensate for each other as fascin creates thick bundles that provide rigidity and strength to invadopodia, while L-plastin-dependent bundles are thin, less packed and promote flexibility of the elongating invadopodium core [450].

A lot can be learned about invadosomes and their higher order structure also by looking at how they relate to actin-based protrusions other than the well-studied lamellipodia and filopodia. For example, invadosome rosettes share many features with circular dorsal ruffles (CDRs) which are created by growth factor-stimulated

fibroblasts, smooth muscle cells and epithelial cells [451]. Interestingly, the same types of cells that display CDRs are capable of creating podosome rosettes. However, as the name indicates, circular dorsal ruffles are created at the dorsal side of the cells, which means that they do not meet rosettes confined to the basal membrane. CDRs are important for processes such as micropinocytosis, receptor internalization and cell motility [452, 453]. In contrast to invadosome containing rosettes, CDRs are not adhesive [451], which could suggest that they are free to move faster. However, both podosome rosettes and CDRs show similar dynamic behavior at a timescale of minutes [313], including for example oscillatory behavior and annihilations upon collision [302, 454, 455]. It means that both of these structure share features of waves propagating in an excitable medium. The same description can be for example used for impulses circulating within the nervous system [302, 454]. Other interesting actin-based structures that share features with invadosome rosettes are ventral F-actin rosettes described in growth factor-stimulated breast cancer cells [343]. These non-degradative cortactin-rich rosettes correlate with membrane protrusions similarly to invadosome rosettes created upon LPA stimulation (**Chapter 3** & **5**), but interestingly they do not contain invadopodia. The lack of invadopodia within these rosettes is surprising as this kind of invasive adenocarcinoma cells spontaneously produce invadopodia at their ventral membrane.

It is not uncommon that different actin-based structures cooperate with each other. Examples of such cooperation include ruffles that protrude from the extending lamellipodium [456], podosomes that form preferentially in the lamella, which is an actin-rich region that localizes directly beyond the lamellipodium [218] or actin waves that accompany ventral lamellipodia [289]. Along this line, in **Chapter5** we show that LPA-induced invadosome rosettes contain a PIP_3-rich structure with all the characterstics of ventral lamellipodia. We demonstrate that the formation of this ventral lamellipodium strongly correlates with the expansion and directional movement of rosettes. Undoubtedly, future studies will unravel new surprising connections between invadosomes and other actin-based structures. Moreover, our growing understanding of the molecular organization of invadosomes (**Chapter 2**) and actin–rich structures in general will hopefully provide a mechanistic insight into the nature of these connections.

Cellular protrusions are not only built up from the actin cytoskeleton. For example, microtentacles are structures of circulating tumor cells that depend on microtubules [457]. It has been shown that these structures enhance the metastatic potential of breast tumor cells by helping them to reattach to endothelial cell layers [457]. Interestingly, invadopodia and microtentacles are differentially regulated by Src kinase [458]. While invadopodia require activity of Src for proper functioning, microtentacles are produced upon inhibition of Src [458]. This thus presents a

clear example of differential regulation of two kinds of cellular protrusions that both are implicated in invasive behavior.

Invadosomes and mechanical signaling

In **Chapter 2** we describe not only the actin organization of invadosomes but also how these structures respond to the mechanical features of the environment. Mechanobiology is a dynamic field of study that thrives at the interface between biology, physics and material sciences. During preparation of this thesis, several interesting studies were published that strengthen the link between formation and function of invadosomes and mechanical properties of cellular microenvironment. For example, using microfluidic devices it was shown that endothelial cells, that can form rosettes upon growth factor stimulation, form instead podosome clusters when placed in confined slits ($\sim$3 μm high) [459]. The same cells formed small podosome rosettes upon entering or exiting the confined space, suggesting that a switch between invadosome clusters and rosettes may not only be controlled by chemical signaling (**Chapter 3**) but also by the forces applied to cells [459]. Furthermore, two detailed review articles have been published recently that summarize our understanding of mechanosensitivity of podosomes [150] and invadopodia [460]. Although multiple lines of evidence suggest that invadosomes may be spots of local mechanical signaling, further studies are necessary to directly unravel molecular events within invadosomes subjected to local forces. These future studies will be possible thanks to the creation of the new FRET-based tension sensors [461, 462], to lessons learnt from focal adhesions, the classical mechanosensitive structures [463], and to the development of sophisticated methods to exert force locally on subcellular regions [464]. Moreover, as other actin based structures, for example CDRs, have also been shown to respond to the mechanical properties of the cellular environment [465], it will be interesting to unravel a possible correlation between their behavior and the behavior of invadosomes upon application of different mechanical stimuli.

The palette of invadosome-type structures and Src

Starting with the initial discovery of podosomes and rosettes in Src-transformed fibroblasts [34, 153], macrophages [71] and osteoclasts [152], the number of cell types that have been shown to produce invadosomes has grown significantly in recent years. To name a few rather unexpected examples, the invadosome family has been expanded by podosomes at the neuromuscular junction [466], actin nodules in platelets [467] and actin-rich foci that drive fusion of muscle cell into

a multinucleated myoblast [468]. One thing remains constant for many different types of invadosomes: they depend on Src signaling.

In order to study invadosome rosettes (**Chapter 3** & **5**) we used Src-transformed melanoma cell lines, mainly invasive A375M cells. Through Src transformation, we created a model system that enabled us to learn about signaling and dynamics of invadosomes. For example, we described the role of the Gα_i - PI3K pathway in the formation of invadosome rosettes upon GPCR agonists stimulation (**Chapter 3**). Recent studies suggest that the role of this pathway in invadosome creation is not restricted to the selected model system. For instance, Hwang and colleagues have shown that LPA-dependent invadopodia in PC3 prostate cancer cells also depend on Gα_i signaling [469].

Nevertheless, the choice for Src-transformed cells has been debated. Although it has been repeatedly shown that Src is aberrantly activated in many types of cancer, it is rarely due to a genetic mutation [470, 471], and thus using a mutated form of Src may seem artificial. Yet, despite substantial progress in understanding Src-related pathways in cancer, the exact spatiotemporal regulation of Src in invadosomes and invasive migration in general remains elusive [316]. In this respect, introduction of active Src conveniently substitutes for a multitude of Src activating events encountered in the organism. For example, Src may be activated by a lack of balance between receptor tyrosine kinases and cytoplasmic phosphatases [471], by reactive oxygen species signaling [472], by trafficking of Src protein [473] and by miRNAs regulation [474]. New Src activating mutations are also being discovered [475]. Finally, as Src-specific inhibitors (Dasatinib, Saracatinib, Bosutinib, etc.) are actively pursued in clinical trials, evidence emerges on the beneficial role of combination therapies targeting Src simultaneously with other signaling pathways [476]. Thus, studying cooperation of Src signaling with other signaling pathways seems even more important.

LPA and cancer

We unraveled the role of certain GPCR agonists in the rearrangement of invadosome clusters into rosettes in Src-transformed melanoma cells (**Chapter 3** & **5**). LPA is one of the most potent inducers of invadosome rosettes. LPA is also involved in invadopodia production in other cancer cells, like fibrosarcoma cells [65], ovarian cancer cells [66] or prostate cancer cells [469]. However, formation of invadosomes is not the only link between LPA and cancer progression and metastasis. For example, melanoma cells are capable of producing LPA gradients that promote their dissemination [64], while breast cancer cells can stimulate activated platelets to release LPA, which promotes proliferation and cytokine-dependent bone resorption activity of the same cancer cells [477]. Moreover, overexpression

of selected LPA receptors in ovarian cancer cells promotes tumor outgrowth and metastasis [478]. Interestingly, although, overexpresssion of LPA receptors alone does not have a transforming effect on fibroblasts, it potently increases transformation potential of the c-Myc oncogene [479].

Autotaxin (ATX), an LPA producing enzyme, has also been implicated in cancer progression. It has been shown that cancer cells overexpressing ATX form osteolytic bone metastases more efficiently [480]. Furthermore, although ATX overexpressing fibroblasts have limited metastatic potential, introduction of ATX into ras-transformed cells significantly increased tumorigenesis and metastatic potential of these cells [481]. It is noteworthy that the overexpression of both ATX and LPA receptors often augments other pro-oncogenic events [330, 479]. In line with the above studies, we observed that LPA induces production of invadosome rosettes only in cells primed by the overexpresssion of activated Src (**Chapter 3**). Our knowledge of the LPA signaling in cancer progression and metastasis is expanding. Future studies will undoubtedly address the contribution of invadosomes to the increased metastatic potential of cancer cells with upregulated LPA signaling. Furthermore, it will be interesting to investigate whether the newly discovered LPA-producing enzyme GDE4 [482] affects invadosome formation.

Potential of new technologies - development of cgLPA

In **Chapter 4** we presented the development of a new caged form of LPA (cgLPA). Caged LPA is a photolabile compound that remains biologically inert until it is illuminated with near-UV light. Therefore it can be used as a source of active LPA in a tightly controlled spatiotemporal manner in a microscopy experiment. We used cgLPA to look at the creation of invadosome rosettes upon subcellular stimulation and within stable LPA gradients (**Chapter 5**). We discovered that the rearrangement of invadosome clusters into rosettes requires targeted stimulation of LPA receptors and as a consequence of this spatially-restricted signaling, rosettes can develop preferentially at the leading edge of the cells migrating within LPA gradients (**Chapter 5**).

Future applications of cgLPA will undoubtedly include studies of invadosomes, as more cell types are being discovered to produce these invasive structures in an LPA-dependent manner. Moreover, cgLPA may also become a valuable tool to study the nervous system, where the spatiotemporal distribution of different LPA receptors critically defines the response of this complex multicellular system. Photolysis of cgLPA will provide the crucial control over both concentration and spatiotemporal delivery of LPA (see also discussion in **Chapter 4**).

Potential of new technologies - development of siFLIM

In **Chapter 5** we studied the dynamical aspects of the LPA-induced rearrangement of invadosome clusters into rosettes. Immediately after LPA stimulation we observed a very rapid expansion of rosettes, which was followed by a prolonged phase of moderate activity. The fast expansion of rosettes is one of those processes that provide a challenge for microscopy, especially for functional imaging. For fluorescence lifetime imaging of fast cellular processes, the limited sampling rate of a microscope may be the source of artifacts in the acquired data. In **Chapter 6** we present the development of a fast new technique to study such signals: siFLIM.

siFLIM enables following changes in lifetimes of fluorophores in fast paced time-lapse experiments. Based on a new phase-sensitive camera (MEMFLIM) and a straightforward calibration step, siFLIM enables acquisition of quantitative lifetime data with speed up to 20 Hz and quality comparable with standard FLIM experiments. We envision that siFLIM will become a valuable tool for biologists to study a whole range of fast cellular processes. In the field of invadosomes, siFLIM could be used to look at the protein interactions in fast moving vesicles that deliver matrix degrading enzymes to podosomes and invadopodia. Moreover, as it has been shown that proteins like paxilin, integrins and cortactin are recruited in a defined manner to both form and disassemble invadosomes within seconds [216], siFLIM has the potential to provide insight into the time-orchestrated interactions of these proteins within those structures.

Concluding remarks

Invadosomes, invadopodia, podosomes, invadosome rosettes and belts together comprise a family of actin-based cellular protrusions that facilitate invasive migration of cells in both normal and pathological processes. As such, they might become targets of new therapies for a range of diseases. However, to control any aspect of cellular behavior successfully, we have to deeply understand the underlying mechanisms. The work presented in this thesis aims to contribute both to our understanding of the signaling pathways of invadosomes as well as to the development of new techniques that may benefit invadosome studies in the future.