Spatial regulation of tumor cell protrusions by RhoC

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Systemic metastasis is the dissemination of cancer cells from the primary tumor to distant organs and is the primary cause of death in cancer patients. How do cancer cells leave the primary tumor mass? The ability of the tumor cells to form different types of actin-rich protrusions including invasive protrusions (invadopodia) and locomotory protrusions (lamellipodia [2D] or pseudopodia [3D]), facilitate the invasion and dissemination of the tumor cells. Rho-family of p21 small GTPases plays a direct role in regulating the actin dynamics in these intracellular compartments. Recent studies have shown that the signaling molecules including RhoC/p190RhoGEF/p190RhoGAP acts as a "molecular compass" in order to direct the spatial and temporal dynamics of the formation of these invasive and locomotory protrusions leading to efficient invasion.

Invadopodia and Lamellipodia: The Invasive Feet

Metastatic dissemination is a major problem in all types of tumors, in which cells colonize distant organs. In breast tumors, in order to leave the primary niche, the metastatic breast carcinomas form a membrane degrading protrusion named invadopodia (**Fig. 1**). Invadopodia, also called "invasive protrusion" to distinguish from "locomotory protrusions" (involved in bulk cell movement), allow the cells to degrade the basement membrane underneath the tissue and penetrate into the tumor stroma. Once in the stroma, tumor cells will migrate within the three-dimensional extracellular matrix. In order to propel themselves in such an environment, tumor cells form actin-rich "locomotory protrusions," also named pseudopodia/lamellipodia (pseudopodia in 3D and lamellipodia in 2D). Once the tumor cells reach the blood vessels, invadopodia facilitate penetration of the tumor cells into the blood stream for tumor cell dissemination.

What are the molecular characteristics of these different protrusions? What extracellular features determine the formation of each of them? How does the tumor microenvironment regulate their formation? In order to understand the molecular mechanisms that drive the formation of the different protrusions, researchers have used many experimental approaches, such as two-dimensional gelatin matrices. In this context, these two types of protrusions can be spatially separated, facilitating at a molecular level the individual analysis of each of them.[1]

Different studies have described that microenvironmental factors such as EGF secreted by macrophages,[2,3] hypoxia conditions,[4] or matrix rigidity,[5] can trigger the formation of invasive protrusions. Among them, EGF secreted by macrophages can trigger the formation not only of invasive protrusions but also locomotory protrusions,[6] facilitating migration and invasion. Stimulation of tumor cells with EGF ligand trigger the formation of invasive protrusions.[7] From various studies we have learned that the formation of an invadopodium is a multistep process composed of a number of well-defined stages: (1) formation of an invadopodium precursor,[7] (2) Tks5-dependent anchoring,[8] and (3) maturation into a degradative structure.[9] Surprisingly, while studying invasive
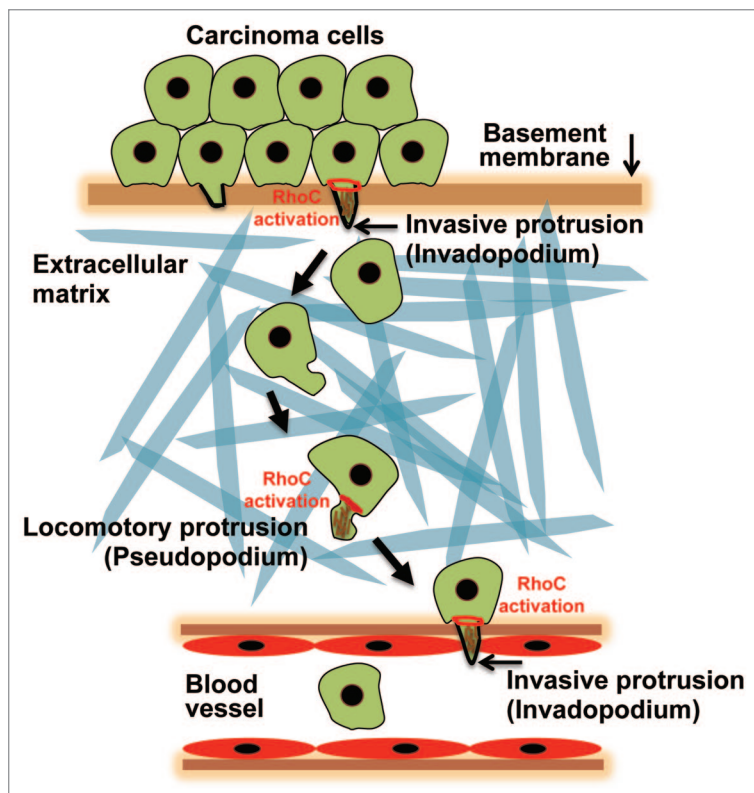
Figure 1. Invasive and locomotory protrusions during tumor metastasis. Tumor cells in the primary tumor form an invadopodium to degrade the basement membrane and penetrate into the tumor stroma. Migration in the three-dimension extracellular matrix is facilitated by locomotory protrusions, such as pseudopodium. When close to the blood vessel, tumor cells form invadopodia in order to penetrate into the blood stream. RhoC (red areas) activation is required to confine protrusion formation.

structures, we have found that some of the key regulatory molecules affecting actin cytoskeleton dynamics such as cofilin or cortactin are shared between those compartments. Invadopodia in addition contained actin-regulatory molecules that associated with filopodia.[10,11] Thus, the question becomes: are locomotory and invasive protrusions similar structures but located at different subcellular locations? Is there a common signaling pathway that determines where they will be formed?

Recent work has shown that the spatiotemporal dynamics of activation of RhoC GTPase plays an important role in confining the actin polymerization in these protrusions facilitating tumor cell migration and invasion.[12,13] The use of high-resolution FRET imaging has revealed that the dynamics of RhoC activity is a common regulator within both compartments; acting in such a way as to geometrically confine and define the location of actin polymerization to affect an efficient

locomotory and invasive protrusion. What are the signaling pathways controlled by RhoC that are shared in both of these compartments?

The Cofilin Activity Cycle at Tumor Cell Protrusions

Cofilin is an actin-binding protein that can regulate actin dynamics in a number of ways.[14] In highly invasive tumor cells it has been shown that cofilin can sever actin filaments to generate new barbed ends that are accessible to monomers of G-actin promoting actin polymerization at invasive[7] and locomotory protrusions.[15] An important step in the cofilin activity cycle is its inactivation step. This step is dependent on the phosphorylation on a serine residue of cofilin, targeted by the ROCK/LIMK pathway. This phosphorylation step takes place after cofilin is released from its inhibitory partner, cortactin, through cortactin phosphorylation[7] by Arg[16] and

Nhe1 activation.[17] These pathways need to be carefully regulated in order to regulate the steps of invadopodium assembly and maturation.

ROCK is a well-known effector of Rho GTPases regulating many pathways related to motility, having greater affinity for RhoC than for RhoA.[18] Inhibition of ROCK by pharmacological inhibitors prevents invasion of breast tumor cells.[19] But which Rho isoform is triggering the cofilin phosphorylation in highly invasive tumor cells? This signaling pathway is under the specific control of RhoC but not RhoA in these metastatic cells. Depletion of RhoC impacts cofilin phosphorylation but on the contrary, RhoA depletion has no effect.[12,13]

These results point to the high degree of specialization of function of GTPase isoforms in tumor cells. We found that RhoC is essential to control cofilin-dependent barbed ends through the activation of ROCK/LIMK at those specific subcellular locations but not RhoA. This cofilin inactivation step triggered by RhoC is crucial for the geometric confinement of active cofilin either at the tip of the leading edge[13] or within the core of the invadopodium,[12] localizing barbed-ends, and therefore, the geometry of actin polymerization in those structures.

RhoC as a "Molecular Compass"

RhoC is necessary for tumor cell invasion. Depletion of RhoC has been shown to affect invasion in many tumors.[12,20,21] How is RhoC regulating invasion at the cellular level? By looking at the ultrastructure of invadopodia we found a striking result when depleting RhoC. While control cells form invadopodia that are capable of penetration into the extracellular matrix, cells depleted for RhoC form abnormal invadopodia structures with multiple branches that cannot efficiently penetrate into the extracellular matrix.[12] Based on this result, we proposed that the defect in confining actin polymerization within invadopodia protrusions might account for the impaired tumor cell invasion after RhoC depletion.

Moreover, RhoC also plays an important role in facilitating tumor cell migration as shown in other studies.[22] In the case of invasive breast tumor cells, the

presence of a local EGF source induces the formation of an actin-rich directional protrusion toward the source of EGF. Depletion of RhoC abolishes the formation of this directional protrusion, and as a result, chemotaxis is impaired.[13] Other studies have indentified RhoC as an effector of integrin signaling[23] and have shown that it is involved in regulation of integrin trafficking in pancreatic tumor cells.[24]

In other cancer models,[21,25-27] and also in cancer stem cells,[28] RhoC has been identified as an important regulator of metastasis. RhoC, through controlling one molecular mechanism (the cofilin pathway) at two specific protrusive compartments, regulates migration and invasion of breast tumor cells. The spatially and temporally specific activation dynamics of RhoC is thus necessary to properly localize and to control these protrusions during invasion and metastasis. Thus, these results could potentially explain why RhoC knockout mice in a MMTV tumor model while forming primary tumors, metastasizes less,[20] and may explain the importance of RhoC in other tumor models. RhoC is also expressed in human macrophages,[29] so it is possible that RhoC may play a role in the regulation of podosome formation, structures also involved in matrix degradation.

Illuminating GTPase Signaling: FRET-Based Biosensors

The signaling pathways that regulate invasive and locomotory protrusions must be well-regulated in space and precisely timed. We have observed that during invadopodium formation, molecules regulating different stages of invadopodium assembly are recruited and activated with a detailed precision during the invadopodium lifetime.[8] So, in this highly regulated series of events: when and where is RhoC activated during their formation?

When studying RhoGTPase signaling, we must take into account that the activity cycling of the Rho GTPases require finely tuned spatiotemporal coordination occurring at seconds resolution and in spatial resolution of sub-microns. FRET-based fluorescent biosensor technology is the ideal technique in order to decipher such rapid dynamics.[30] To answer the question of where and when RhoC is activated, we
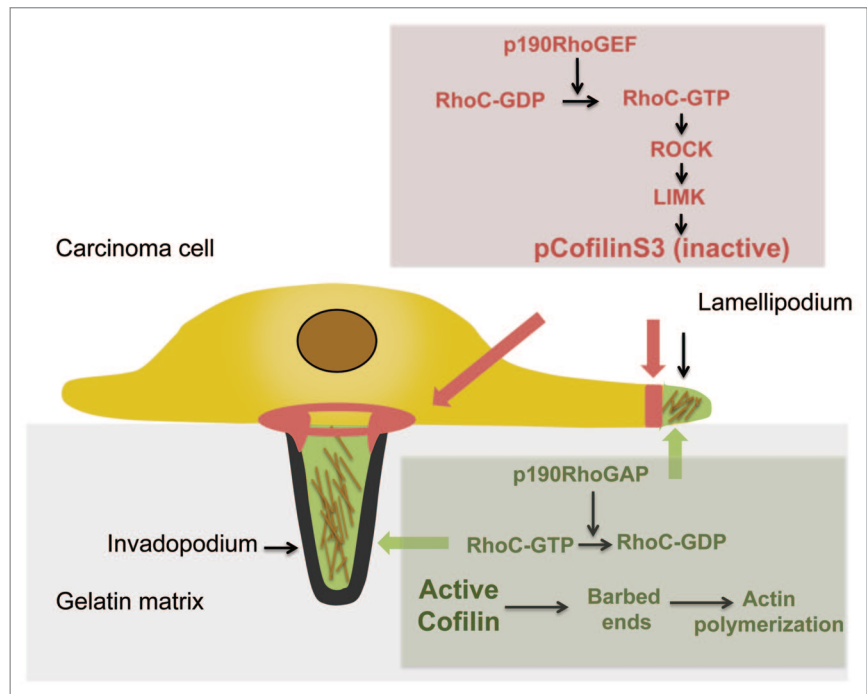

Figure 2. Spatial location of RhoC at invasive and locomotory protrusions and upstream and downstream pathways. p190RhoGEF activates RhoC outside of the invadopodia or behind the cell edge (red areas). RhoC activates the ROCK/LIMK pathway that inactivates cofilin, through phosphorylation (pathway detailed in red box). p190RhoGAP localized at the tip of the lamellipodium or the core of the invadopodium inactivates RhoC (green areas) confining cofilin activity, barbed end formation, and actin polymerization for efficient protrusions (pathway detailed on green box).

used a recently developed RhoC biosensor[31] that allows the study of the activation of RhoC during tumor cell protrusion formation.

The results obtained with this technology allowed us to better understand the functional data obtained using RhoC depletion and other traditional biochemical means. RhoC activity spatially and temporally surrounds the invadopodium core and localizes behind the lamellipodium at the leading edge of migrating tumor cells. The activation of RhoC at those areas triggers the ROCK/LIMK pathway phosphorylating cofilin and geometrically confining cofiln activity, barbed end formation, and actin polymerization at the core of the invadopodium and at the tip of the leading edge of the lamellipodium (**Fig. 2**).

RhoC Upstream Regulation: p190RhoGEF/p190RhoGAP Takes the Lead

While only three close isoforms for Rho, RhoA, B, and C are known, over

70 GEFs[32] and 70 GAPs[33] are known to regulate RhoGTPases. This molecular organization of the RhoGTPase signaling suggests that what could determine where and when GTPases are activated/inactivated, must be the spatial and temporal regulation of the upstream molecules. We have shown that at invasive and locomotory protrusions, RhoC is regulated by p190RhoGEF/p190RhoGAP.[12,13] These two molecules display very specific localization patterns that contribute to confine the activation of RhoC at specific areas to control the cofilin pathway and confine actin polymerization (**Fig. 2**).

In our studies we found that, during lamellipodium protrusions, other GEFs including LARG, p115RhoGEF, or the GAP DLC-1, do not show the same localization pattern as p190RhoGEF/ p190RhoGAP and do not affect cofilin phsophorylation.[13] How are these GEFs and GAPs placed at specific locations and activate/deactivate Rho GTPases only under specific conditions? We can hypothesize that factors including the membrane contour, specific scaffolding

protein organization at the plasma membrane, assembly of focal adhesion components, and activation of upstream kinase pathways, could be responsible for the recruitment of these molecules that can be activated prior to the GTPases being delivered to these particular locations.

By interfering with p190RhoGEF and p190RhoGAP, the amount of barbed ends during lamellipodium protrusion can be modulated through cofilin phosphorylation triggered by RhoC.[13] But interestingly, the signaling module composed of p190RhoGEF/p190RhoGAP/RhoC must be present at all times in order to form efficient protrusions. By interfering with the components, protrusion formation is abolished.

RhoA and RhoC: The Good and the Bad Twin

RhoA and RhoC share almost 88% of their primary sequence. While they both regulate the actin cytoskeleton, their roles in invasion seems to be quite different.[34] While RhoA inhibits invasion, RhoC promotes invasion and metastasis.[20,22,35] Strikingly, when looking at the activation of RhoA in relation to RhoC, we found that at invasive protrusions, RhoA does not seem to display any specific activation patterns, while at locomotory protrusions, RhoA is activated in a narrow band at the edge of cell protrusions. These different spatial localization patterns of RhoA in comparison to RhoC suggest different roles each isoform of Rho GTPases may play during these physiological processes. These different subcellular localizations, specifically at the leading edge, seem to be a conserved feature since in fibroblasts the analysis of RhoA and RhoC activities revealed similar findings.[31] Recently, Machacek et al.[36] has shown that RhoA, Rac1, and Cdc42 display highly characteristic and specific activation patterns during leading edge protrusions in fibroblasts. The coordinated dynamics of the Rho GTPase activations at the protrusions may likely require and affect potential feedback/forward mechanisms activating/repressing certain GTPases, as has been shown for RhoA/Rac.[37]

While RhoA plays an important role in delivering MT1–MMP[38] to invadopodia,

RhoC is involved in regulating the actin cytoskeleton through the cofilin pathway. By contrast, at locomotory protrusions, while RhoC is important for the directional polarization and formation of protrusions, RhoA does not seem to be necessary. Cells depleted for RhoA can still form actin-rich protrusions.[13] Interestingly, at locomotory protrusions when the lamellipodium compartment is removed, a RhoA–mDia1-dependent pathway is activated,[39] and in that situation we were able to localize high levels of activation of RhoA reveling the role of RhoA in regulating these mDia1-dependent filaments at locomotory protrusions.[13]

These studies show the importance of observing signaling pathways and GTPase activations at subcellular spatial resolutions and in time scales of seconds, thus the use of FRET-biosensors is the ideal approach for these studies.

Conclusions

We are only starting to understand how RhoGTPases are regulated at locomotory and invasive protrusions and the relationship between these two actin-rich protrusions at a molecular scale. By identifying the molecules and the signaling pathways that commonly regulate these different protrusions, we will be able to define critical pathways that could be targeted for possible intervention to halt metastasis.

Important questions remain: (1) what recruits GEFs and GAPs to their specific locations; (2) how are GEF/GAP activities regulated; and (3) what regulates the specificity of each GEFs and GAPs? Clearly, far more work is needed to address these critical issues in order to better understand the mechanisms by which isoform-specific roles of GTPases are regulated, both within specific tumor microenvironments and from the metastatic context in vivo. The development of new imaging technologies, including FRET-based biosensors to monitor the activation of the upstream molecules, will certainly shed light onto these aspects, as well as development of approaches to directly visualize protein activation dynamics in live animals in vivo will clear the way to understanding how protein-activation level events regulate the dynamics of tumor dissemination. We are

in a very exciting time when the technology is moving fast enough to potentially address all these questions and better our understanding of tumor metastasis.

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