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RhoA can lead the way in tumor cell invasion and metastasis

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1. ABSTRACT

The Rho family of GTPases is well-established regulators of cell migration, and has been implicated in the process of tumor cell invasion and metastasis. The RhoA signaling pathway is strongly correlated with the ability of tumor cells to invade and successfully establish metastases. In this review, we begin by discussing the gene expression data correlating Rho expression with metastasis, and then discuss two emerging concepts that help explain the underlying mechanisms by which RhoA may promote tumor metastasis. First, the use of sophisticated biosensor probes has revealed that RhoA is active in membrane protrusions. Second, the RhoA pathway affects the invasive behavior of tumor cells by promoting invadopodia, amoeboid migration, and the plasticity of tumor cells to modulate their migratory properties. Thus, our view of the role of the RhoA pathway in metastasis is evolving to include a previously unappreciated function at the leading edge.

2. INTRODUCTION

The Rho family of small GTPases, comprised of Rho, Rac, and Cdc42, were discovered in the 1990's and found to be potent regulators of the cytoskeleton (1). As such, they are essential regulators of cell migration and invasion, contributing towards diseases related to cell migration including cardiovascular disease, dysregulated inflammation, and metastatic cancer (2-5). Traditionally, the Rac and Cdc42 branches of the Rho family have been associated with protrusion and forward motility, while the opposing Rho branch was associated with inhibition of protrusion and promotion of large actin bundles and integrin adhesion complexes (6). Thus, it was originally thought that high Rho activity would inhibit invasiveness, but this notion was challenged by the findings that RhoA expression has a strong positive correlation with invasion and metastasis. Here we will review the mounting evidence supporting a strong positive association between RhoA expression and metastasis, and then discuss recent studies that provide the underlying mechanistic

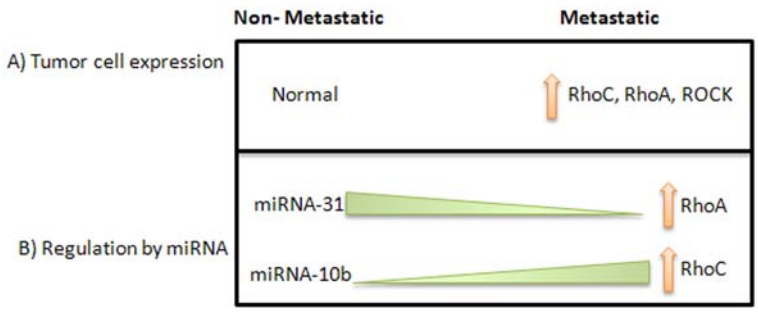

Figure 1. Rho GTPase and ROCK expression has strong positive correlation with metastasis. A) The relative expression of RhoA, RhoC and ROCKs in cell lines with different metastatic potential has been determined by micro array analysis or RT-PCR. RhoA, RhoC, and ROCKs have increased expression in the metastatic variants. B) miRNA-31 and miRNA-10b regulate the metastatic potential of tumor cells. Loss of miRNA-31 is associated with increased metastasis and concomitant increase in RhoA expression. Conversely, increased expression of miRNA-10b enhances the metastatic potential of tumor cells, which is associated with increase in RhoC expression.

explanations. Tumor cell migration models have undergone significant evolution in terms of our view of RhoA signaling as we now realize that its activity at the leading edge may be critical for its ability to drive invasion and metastasis.

3. RHO EXPRESSION IN METASTASIS

3.1 RhoC expression is positively correlated with metastasis

Progression of tumor towards metastasis is often depicted as a multistage process in which malignant cells migrate from the tumor of origin and colonize in distant organs (7). With the use of transcriptomic microarray analysis, patterns of gene expression associated with aggressive metastatic behavior have been identified (8-12). Enhanced expression of several genes involved in extracellular matrix assembly and genes that regulate, either directly or indirectly, the actin-based cytoskeleton have been identified as pro-metastatic factors (Figure 1) (8, 9, 13).

One of the earliest screens to compare gene expression between metastatic and nonmetastatic cells used an *in vivo* selection scheme to isolate highly metastatic tumor cells. In this study, weakly metastatic cells were intravenously injected in nude mice and the lung metastases were dissected. Cells of metastatic colonies were then expanded in tissue culture and reintroduced into host mice. This procedure was repeated three times and cell populations with high metastatic potential were isolated. Gene expression was compared between the high metastatic potential populations and the parental populations with the use of microarray analysis. Fibronectin, RhoC, and thymosin β4, were the three genes that were highly expressed in the metastatic variants. The importance of RhoC in metastasis was confirmed by overexpressing RhoC in the weakly metastatic parental cell lines and demonstrating that RhoC was sufficient to cause metastasis to lungs. Interestingly, cells overexpressing RhoC did not show increased proliferation, but instead showed a more invasive and elongated morphology, along with increased migration. This work was the first to identify the significance of RhoC in metastasis (10).

3.2. MicroRNAs regulate tumor metastasis by modulating Rho expression

The discovery of more than 650 microRNAs in the human genome as regulators of gene expression has led to the development of microRNA genetic screens (14). MicroRNAs (miRNA) are small, noncoding RNAs that negatively regulate gene expression. miRNAs can regulate tumor development, progression, and metastasis by functioning as either a tumor promoters or tumor suppressors. miRNA screens of nonmetastatic and metastatic tumor cell lines have identified miRNA-31 (miR-31) as a repressor and miR-10b as a promoter of tumor metastasis. Valastyan *et al.* found that overexpression of miR-31 suppresses metastasis in otherwise aggressive breast tumor cells, and that inhibition of miR-31 expression *in vivo* allowed non-aggressive breast cancer cells to metastasize (12). Analysis of different steps during the metastasis process showed that miR-31 inhibited local invasion, extravasation, and metastatic colonization. At the gene expression level, miR-31 was found to coordinately repress a cohort of metastasis-promoting genes including RhoA. The importance of miR-31-mediated repression of RhoA was confirmed as reexpression of RhoA partially reversed miR-31 mediated metastasis suppression (12, 15).

In a separate study, miR-10b was found to be significantly overexpressed in metastatic breast cancer cells. miR-10b was shown to promote cell invasion and migration *in vitro* and act as an initiator of tumor invasion and metastasis *in vivo* (11). Ma *et al.* shows miR-10b directly inhibits translation of HOXD10, a homeobox domain protein that normally acts to repress the expression of several genes involved in cell migration. The pro-metastatic activity of miR-10b was due to increased expression of these HOXD10-regulated genes, including RhoC. Importantly, depletion of RhoC was sufficient to block the pro-invasive effects of miR-10b overexpression.

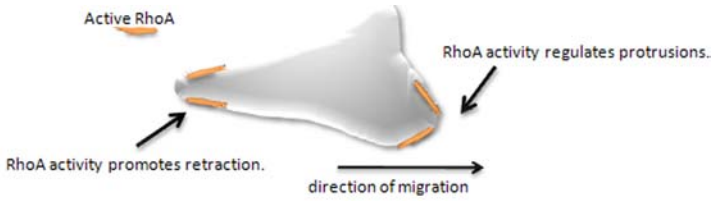

Figure 2. Localization of RhoA activity in migrating cells. FRET biosensors that specifically measure active GTPases were used to determine the spatiotemporal localization of RhoA in cells migrating on a 2-dimensional surface. As expected, active RhoA can be found at the back end of the cell during a retraction event; however, active RhoA was also detected in membrane protrusions at the leading edge.

These unbiased screens establish that expression of RhoA and RhoC act as metastasis promoters and implicate miRNAs as regulators of their expression. The correlation between RhoA and RhoC expression with metastasis has been confirmed with several studies surveying gene expression in both tumor cell lines and clinical tumor samples (16-18). Further, a causal role for RhoA and RhoC with metastasis has been demonstrated by siRNA-mediated inhibition of RhoA and RhoC, which blocked the invasiveness of breast cancer cells *in vitro* and *in vivo* (19). Substantial evidence linking RhoA and RhoC to metastasis now exists, and has spurred significant research effort to understand the mechanisms by which the RhoA GTPases can lead to the invasion of malignant tumor cells.

4. THE RHOA PATHWAY AT THE LEADING EDGE

Tumor metastasis is accompanied by changes in migratory properties as the result of reorganization of the cytoskeletal architecture. Rho GTPases are well-established as essential signaling molecules that coordinate the dynamics of cytoskeletal rearrangements in space and time during migration (6). Until recently, the functions of individual Rho GTPases were largely inferred from studies where its activity was manipulated by expressing specific point mutants. While these studies have provided a wealth of information regarding Rho GTPases in regulating distinct steps of migration, critical information regarding the spatiotemporal regulation of these molecules was lacking.

4.1. Spatiotemporal activation measured with biosensors

Development of fluorescence resonance energy transfer (FRET) biosensors specifically designed to detect activated GTPases has greatly advanced our knowledge of signal transduction in live cells (20). With the use of FRET biosensors it is possible to study the highly dynamic spatiotemporal kinetics of these GTPases previously missed by biochemical assays. Previous studies predicted spatial segregation of RhoA and Rac activity. RhoA was predicted to be active at the back of the cell to promote retraction, while Rac would be active at the cell front to promote protrusion. Quite unexpectedly, studies using activation-dependent FRET probes have established that active RhoA also localizes to the leading edge of migrating cells (21-23). This suggests that in addition to its well-established role in tail retraction, RhoA also regulates protrusion at the front of the cell. To assess the functional role of these GTPases at the leading edge, "computational multiplexing" was used to show that Rac and Cdc42 are less spatiotemporally coupled to initial protrusion than is RhoA. Interestingly, increases in RhoA activity were correlated with increases in protrusion rates and was synchronous with cell edge advancement (23). These unexpected findings demonstrate that localized RhoA signaling is functionally associated with membrane protrusions, and substantially altered our view of its role in migration by establishing its importance in leading edge dynamics (Figure 2).

4.2. Upstream activators at the leading edge

Following the discovery of active RhoA at the leading edge, a current research objective is to identify the upstream activators that are involved in the spatiotemporal activation of RhoA. It is possible that depending upon the extracellular signal, upstream activators of RhoA can govern the spatiotemporal activity of RhoA in a very context-specific manner. Recently, a microtubule associated Rho GEF, GEF-H1, was shown to stimulate localized RhoA activity at the leading edge (24). FRET biosensors were used to show that depletion of GEF-H1 by siRNA specifically blocked activation of RhoA at the leading edge while leaving overall RhoA activation unaltered. To determine the function of RhoA activity at the leading edge, several cytoskeletal parameters were assessed. Depletion of GEF-H1 led to altered organization of the actin cytoskeleton, disrupted membrane dynamics, and decreased focal adhesion turnover. Importantly, the disruption of RhoA-dependent cytoskeletal dynamics at the membrane was accompanied by inhibition of migration. Thus, GEF-H1 controls the spatiotemporal activation of RhoA at the leading edge of a migrating cell.

Another study focused on the exchange factor, MyoGEF, which is highly expressed in metastatic breast tumor cells and is localized to acto-myosin filaments at the leading edge (25). Biochemical studies found that the substrates of MyoGEF are RhoA and RhoC, identifying another Rho GEF that may selectively activate RhoA at the front of the cell. Significantly, knockdown of MyoGEF blocked invasion suggesting MyoGEF-mediated activation of RhoA or RhoC at membrane protrusions may promote invasive behavior of tumor cells. These studies suggest that multiple GEFs are associated with localized activation of RhoA at the leading edge and suggest a model where specific GEFs are used to differentially translate cues from

the microenvironment to coordinate the spatiotemporal regulation of RhoA activity in membrane protrusions and promote cell invasion and migration.

4.3. RhoA GTPase effectors at the leading edge

The next step in understanding RhoA function at the leading edge is to determine which downstream targets mediate its function at the membrane. RhoA binds to effectors via a Rho binding domain (RBD) and its effectors can be divided into three groups according to RBD subtypes (26). A common mechanism of activation is the induction of a conformational change in the effector upon RhoA binding. This allows the effector to induce cytoskeletal rearrangements and regulate cell migration and invasion. Of all of the effectors, the majority of data is focused on the ROCK and formin proteins; thus, we will focus our discussion on these two effector families, but is should be noted that the potential for other RhoA effectors in invasion and metastasis remains open.

4.4. Formins as RhoA effectors

mDia was discovered in 1997 as a RhoA effector through a yeast 2-hybrid screen and shown to be localized with RhoA at the cell periphery and induce F-actin structures in cells (27). mDia is now recognized to be a member of a large family of related proteins known as formins, a subset of which contain Rho binding domains. Studies to determine the *in vivo* role of mDia proteins are limited, but demonstrate that mDia1 regulates hematopoietic cell proliferation and migration (28, 29). *In vitro* studies using knockout mouse embryonic fibroblasts, siRNA, or mutational analysis show that mDia can be localized to the leading edge, where it can regulate actin polymerization, microtubule stability, and adhesion dynamics (30). Together, there is substantial evidence to support formins as downstream effectors of RhoA at the membrane.

Multiple formin proteins have been shown to stimulate nucleation and polymerization of actin filaments through their formin homology 1 (FH1) and formin homology 2 (FH2) domains (30-32). The FH1 domain binds to profilin, a protein that binds to monomeric actin and facilitates its incorporation into growing actin filaments. The FH2 domain binds to the barbed ends of actin filaments and moves processively along the growing filament, stimulating rapid elongation by protecting the end from capping proteins. Formins are associated with multiple cellular structures including actin stress fibers, lamellipodia, and adherens junctions between cells; thus, their actin polymerizing activity can be harnessed to form diverse populations of actin structures. Because actin polymerization is thought to be a primary driving force in membrane protrusion, it is likely that formins are important targets for RhoA signaling in the regulation of membrane protrusion at the leading edge.

In addition to their initially described role in actin polymerization, formins also play a key role in regulating microtubules. mDia stabilizes microtubules, producing subsets of stabilized microtubules that tend to localize near the leading edge (33, 34). Formins can stimulate microtubule stabilization in response to diverse upstream signals including LPA stimulation or integrin engagement (33, 35). Interestingly, Palazzo *et al* also demonstrated that membrane targeting of RhoA was required for microtubule stabilization, suggesting spatial regulation of RhoA is essential for coupling to its downstream effectors. While the mechanism responsible for mDia stabilization of microtubules is not completely understood, it is known to bind to several different microtubule tip proteins, including EB1 and APC (36). Because microtubules are important for determining the polarity and directionality of migrating cells, formins can promote cell migration by regulating microtubule behavior. It also suggests the possibility that because formins interact with both actin and microtubules, this effector pathway may be involved in crosstalk between these two filamentous cytoskeletal systems to regulate the behavior of the leading edge.

4.5. ROCKs as RhoA effectors

ROCKI and II are serine/threonine kinases that were first identified as RhoA targets in the mid 1990's, and subsequently shown to significantly stimulate the formation of actin stress fibers and integrin adhesion complexes through myosin contractility in fibroblast cells (37, 38). ROCK is now recognized as a multi-functional kinase, impacting a wide range of physiological functions, including the nervous system, the cardiovascular system, and stem cell mobilization (4, 39-42). ROCKs regulate the recruitment of cells during inflammation or stem cell mobilization, are required for cell division, and impact tissue organization during neovascularization and tumor progression. Various animal models have established that ROCK activity is required for the metastasis of tumor cells. Recently, it was reported that increased levels of ROCK1 were significantly correlated with human mammary tumor development and increased tumor grade (43). Increased ROCKI and ROCKII expression was also observed in bladder cancer patients and was positively correlated with metastasis (44). Several lines of investigation also implicate ROCKs in the invasive behavior of tumor cells (44-50). Thus, ROCKs have proven to be potent regulators of cell migration and invasion both *in vitro* and *in vivo*.

ROCKs have well-established functions at the back end of migrating cells to promote retraction of the tail, but its potential role at the leading edge has not be thoroughly investigated (51, 52). However, ongoing studies in our laboratory suggest that ROCKII is an important effector of RhoA function at the membrane (unpublished data). We expressed GFP-ROCKII in MCF-7 cells and found that GFP-ROCKII localizes to protrusive areas (Figure 3). During these experiments, we found that the localization was transient, indicating that the membrane localization is tightly controlled in response to upstream signaling. Our results are in contrast with the study by Kurokawa *et al*, who used photo-changeable probes to measure mDia1 and ROCK localization at the leading edge, and showed that mDia1 was stably associated with the membrane while ROCK was not (21). One possible explanation for the differences between these findings is that mDia1 is more stably associated with the membrane

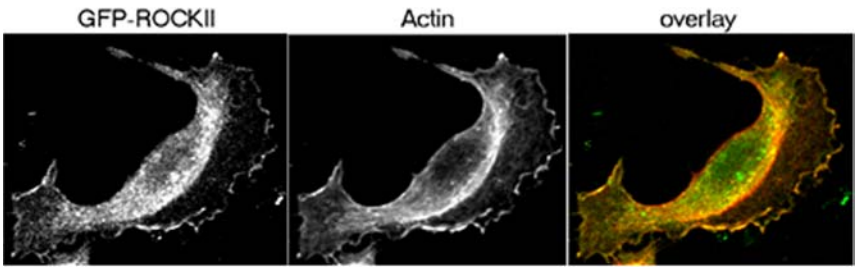

Figure 3. Localization of ROCKII in migrating cells. MCF7 cells expressing GFP-ROCKII were fixed and stained for f-actin. The localization of GFP-ROCKII was determined by confocal microscopy. We observed co-localization between GFP-ROCKII and f-actin in membrane ruffles at the leading edge. This establishes ROCKII as a potential effector for mediating the RhoA signaling in membrane protrusions.

while ROCK association was more transient, or that an appropriate upstream stimulus is required for ROCKII to associate with the membrane. It is also possible that there are isoform-specific localization patterns between ROCKI and ROCKII. The fact that ROCKs are strongly associated with invasive behavior, along with our observations that ROCKII is localized in membrane protrusions, suggests an important role for ROCKs as RhoA effectors at the front end of invasive cells.

To understand how ROCKs might regulate the behavior of the leading edge, we will discuss their downstream targets. ROCKs have multiple substrates, the best known being the phosphorylation of the myosin II light chain, MLC (41, 53). ROCKs also phosphorylate and inactivate the myosin phosphatase subunit 1 (MYPT1), leading to an increase in myosin II light chain phosphorylation. The end result is that ROCK activity increases the activity of the myosin motor, promoting the contractile force being applied to the actin filaments that are in contact with myosin. Myosin II contractility is critical for multiple facets of cell migration, including the retrograde flow of actin in the lamella and in the maturation of integrin adhesion complexes (54, 55). Thus, membrane localized ROCK could contribute to leading edge dynamics during cell migration through its effects on myosin II activity.

ROCKs are also known to phosphorylate and activate Ezrin/Radixin/Moesin (ERM) proteins (41, 56). These proteins have a C-terminal actin binding domain and an N-terminal membrane binding domain, making them linkers between the membrane and the cortical actin cytoskeleton. It is thought that ERM proteins regulate the function of transmembrane or cell surface receptors by organizing their function in specific membrane microdomains (57). ROCK activity is linked to the localization of ERM proteins to membrane, and in a 3-dimensional tumor invasion model ROCK activity was required for ERM proteins to be specifically localized to the invading front (58). Ezrin, in particular, has been linked to tumor progression and metastasis both in humans and in animal models (59). Several integrins bind to ERM proteins, suggesting that the correlation of ezrin with migration and metastasis may be through regulation of adhesion receptors at sites of protrusion. In this way, ROCKs could work through ezrin to control the interaction of tumor cells with the microenvironment, to mediate tumor cell invasion and recruitment during metastasis.

5. INVASIVE BEHAVIORS REGULATED BY THE RHOA PATHWAY

Tumor cell invasion and metastasis is a multistage process. A metastasizing tumor cell must break free of the primary tumor, invade and migrate through basement membrane and interstitial matrix, traverse blood vessels, and colonize a distal site. Such a formidable task likely requires the utilization of multiple modes of cell migration and invasion. This has been highlighted by recent studies focused on the migration and invasion of tumor cells in 3-dimensional systems. In some tumor cells, invasion through a 3-dimensional matrix is led by invadopoda, while other tumor cells navigate through 3-dimensional substrates using an amoeboid style of migration that is driven by membrane blebbing. The strong correlation between RhoA and ROCK with tumor cell metastasis may stem from the fact that RhoA signaling can regulate both invadopodia-driven and bleb-driven amoeboid invasion (Figure 4).

5.1. RhoA GTPase pathway promotes invadopodia

Invadopodia are small, actin-rich membrane protrusions that are observed in invading tumor cells. These podosome-like structures are enriched in cell-matrix adhesion molecules, integrins, and actin regulatory proteins; in addition, they are capable of localized extracellular matrix (ECM) degradation due to active exocytosis of proteolytic matrix metalloproteinases (MMPs) (60-62). RhoA is localized to invadopodial structures and its activity is essential for invadopodia formation, regulation, and invasive potential. RhoA activity is required for formation of invadopodia, as inhibition with either C3 or dominant-negative RhoA disrupts the accumulation of f-actin and its associated invadopodia marker, cortactin. RhoA inhibition also dramatically decreases MMP secretion suggesting Rho activity is also required for the degradative potential of the invadopodia (63). This is supported by Sakurai-Yageta *et al*, who confirmed RhoA as critical for invadopodia formation and MMP secretion (62). The authors determined that RhoA acts in concert with cdc42 to

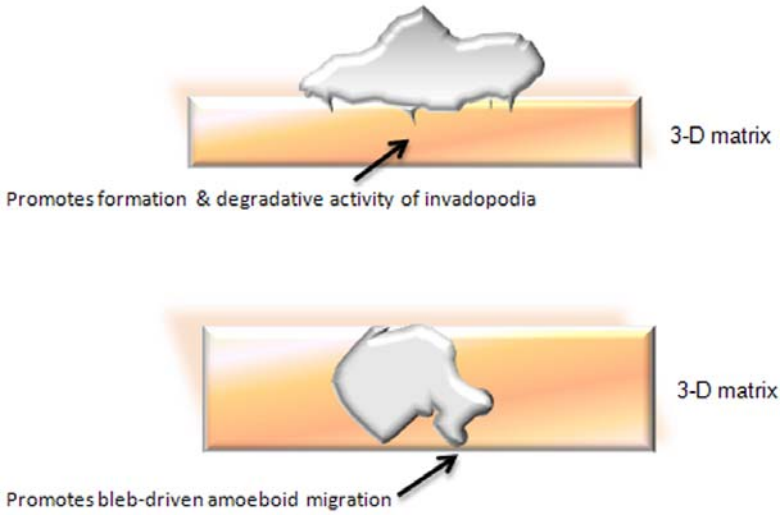

Figure 4. RhoA signaling pathway in invasive behaviors of tumor cells. The RhoA signaling pathway regulates multiple types of invasive behaviors exhibited by tumor cells. The particular mode of migration depends on the tumor cell type. TOP: Invasion through basement membrane by invadopodia, typical of MDA-MB-231 cells. RhoA signals through both ROCKs and Formins to regulate both the formation of invadopodia and its degradative capacity. ROCKs and Formins are proposed to regulate the formation of invadopodia through effects on myosin II contractility and actin polymerization. ROCKs have further been shown to regulate the release of metalloproteinases for degradation of the surrounding matrix. BOTTOM: Invasion through 3-dimensional substrates using an amoeboid mode of migration, typical of MDA-MB-435 cells. RhoA signals through both ROCKs and Formins to regulate amoeboid migration that is thought to be driven by membrane blebs. RhoA and ROCK activity are strongly associated with amoeboid migration and the dynamic blebbing through regulation of myosin II contractility. More recently, Formins have also been implicated in amoeboid migration, though the mechanism has not been determined.

promote invadopodia formation and regulates the exocyst complex, where the exocyst facilitates tethering and polarized exocytosis of MT1-MMP-loaded transport vesicles at the site matrix proteolysis and remodeling. Thus, RhoA promotes invadopodia through both regulation of actin dynamics, as well as regulation of protease-loaded vesicle docking and exocytosis.

Together, the above studies demonstrate that that RhoA can promote the invasive behavior of tumor cells through the regulation of invadopodia. While further studies are required to fully understand the spatiotemporal regulation of RhoA in invadopodia and what downstream RhoA effectors modulate the formation and activity of these invasive structures, data suggest there is, indeed, dynamic regulation of RhoA activity at protruding invadopodia. A negative regulator of RhoA activity, p190RhoGAP, localizes to invadopodia where its activation stimulates the membrane protrusiveness of invadopodia (64). Inhibition of p190RhoGAP blocks RhoA-induced invadopodial protrusions, prevents matrix degradation, and inhibits cell invasion. Recently, a novel invadopodia-associated protein, p27RF-RhoA, was identified through its interaction with MT1-MMP and localization in the invadopodia (65). p27RF-RhoA promotes the activation of RhoA at invadopodia by sequestering p27(kip1), which when free, inhibits RhoA activation by RhoGEFs and inhibits metastasis (66, 67).

The RhoA effectors, ROCKs and formins, also regulate invadopodia function. Inhibition of ROCK or myosin II activity with pharmacological agents reduces invadopodia number, suggesting that ROCK-mediated regulation of myosin II-based contractility promotes formation of invadopodia (68, 69). ROCK activity may also be required for the degradative capacity of invadopodia, as several studies demonstrated ROCK is required for MMP activation. In colon cancer cells, siRNA-generated knockdown of ROCKII decreases tumor cell invasion by 2-3 fold with concomitant decreases in MMP-2 and MMP-13 activity (70). This is similar to what others have observed in non-tumor cells lines, where ROCKII has been implicated in the regulation of MMP-2, -9, and -13 (71, 72). Thus, ROCKs are important effectors of RhoA-driven invasive behavior, in part, through stimulation of the degradative activity of MMPs at invadopodia.

The contribution of formins to invadopodia have also been recently identified as knockdown of Dia1, 2, or 3 each were able to block the formation of invadopodia in MDA-MB-231 cells, which was correlated with an inhibition of invasion of this highly invasive cell line (73). These experiments showed that Dia proteins were required for the accumulation of f-actin around invadopodia, suggesting that the actin polymerizing activity of formins contribute to its role in invasion. Furthermore, it demonstrates a role for formin proteins in the invasive behavior of malignant tumor cells. Together, the data show that RhoA may use multiple effectors to regulate the invasive behavior of tumor cells by influencing the formation and degradative activity of invadopodia.

5.2. RhoA GTPase pathway promotes amoeboid-like motility

Tumor cells migrating away from the site of a primary tumor must invade through a dense network of ECM proteins. For many years the primary model of tumor cell invasion through matrix was based on the mesenchymal mode of migration observed on a 2-dimensional surface (74). During mesenchymal motility, cells exhibit an elongated, polarized cell morphology and extend Rac-dependent, f-actin-rich protrusions from the leading edge. Matrix metalloproteinases are secreted to degrade the matrix ahead of the cell path. Rho activation during mesenchymal migration is thought to be relatively low, used primarily to generate cellular contraction to pull the cell forward through the extracellular space using integrin adhesion complexes as sites of traction generation (75).

Recent observations of tumor cells migrating three dimensional matrices, or *in vivo,* revealed that tumor cells can migrate and invade extracellular spaces with an alternate form of cell migration termed amoeboid motility, reminiscent to that observed in *Dictyostelium* (75-78). Tumor cells undergoing amoeboid migration typically exhibit a rounded phenotype with high, active membrane blebbing and are not dependent on MMP–generated ECM degradation (77). High RhoA signaling is strongly associated with utilization of amoeboid motility, representing another type of invasive cellular behavior promoted by Rho that likely contributes to the strong correlation between Rho and metastasis.

Current research is focused on discovering how upstream and downstream mediators of the RhoA pathway control this previously unappreciated invasive function of Rho in malignant tumor cells. ROCKs have become well-established, critical mediators of amoeboid migration through their ability to regulate of myosin II contractility. Images from Wyckoff *et al* reveal that during cell invasion, MLC is organized in thick bundles, perpendicular to cell movement and just behind an f-actin-rich leading edge/invading front (79). They show that ROCKI localizes just behind and within this actin-rich invading edge. Phosphorylation of MLC by ROCKI is required for matrix deformation as pharmacologic inhibition of ROCK activity or dominant negative ROCK disrupted orientation and organization of MLC bundles and resultant force generation. The contractile force generated by high RhoA and ROCK activity at the cell cortex creates hydrostatic pressure and generates blebbing of membrane and facilitates cytoplasmic "streaming" through spaces in the ECM as matrix is pushed away in front of it. These data suggest that the Rho-mediated membrane dynamics/blebbing generated by high RhoA-ROCK signaling at the leading edge of an invading tumor cell is an essential component in the regulation amoeboid migration and invasion.

While the role of ROCKs are more well-established in amoeboid motility of tumor cells, it was recently determined that formins can also stimulate this style of invasive migration. When examining MDA-MB-435 cells, it was found that Dia1 is part of a positive feedback loop where it can stimulate the Rho exchange factor, LARG, thereby further activating RhoA and ROCK (48). In this way, Dia1 was able to promote the invasion of MDA-MB-435 cells. In this study, Dia1, and not Dia2, was required for invasion, which is in contrast to a previous study where both Dia1 and 2 were able to block invadopodia and matrix invasion (73). One possible explanation is that one of the studies utilized MDA-MB-231 cells, which invade with invadopodia, while MDA-MB-435 cells utilize the amoeboid migration mode. Recently, a siRNA screen was performed to determine which formin family members could modulate tumor cell invasion, and compared the effects between MDA-MB-231 vs. MDA-MB-435. They found that one protein, formin-like protein 2 (FMNL2) was specifically required for the amoeboid style invasion of MDA-MB-435, but did not affect the invasion of MDA–MB-231 cells (80). Interestingly, the screen also revealed that several other formins decreased invasion selectively in the MDA-MB-435 cell line. Thus, it is likely that RhoA may selectively utilize different formin family members for amoeboid migration depending on the cellular context.

5.3. RhoA pathway in plasticity of invasive behaviors

Migrating tumor cells exhibit plasticity for the type of motility they use: MMP proteolysis-dependent cells can convert to an amoeboid mode (mesenchymal to amoeboid transition, MAT) when extracellular proteolytic activity is pharmacologically inhibited, while amoeboid cells switch to a mesenchymal motility (amoeboid to mesenchymal transition, AMT) when RhoA or ROCK activity is inhibited. This plasticity from mesenchymal to amoeboid and amoeboid to mesenchymal motility appears to be dependent on an inverse relationship between Rho and Rac activity (Figure 5) (76, 77, 79, 81-85). Sanz-Moreno *et al.* used a siRNA screen to identify GAPs and GEFs that regulate MAT or AMT in melanoma cells grown on 3D collagen gels (84). They identified DOCK3, a GEF for Rac1, as a key regulator of amoeboid to mesenchymal (AMT) motility. Knockdown of DOCK3 or Rac1 blocked AMT; conversely, overexpression of Rac1 promoted the mesenchymal phenotype. The opposing relationship of Rac1 and RhoA-ROCK activity in determining the mode of migration was further demonstrated by experiments showing that high ROCK2 activity promotes amoeboid motility in A375M2 cells by suppressing Rac1 activity via ARHGAP22, a Rac1 GAP. They concluded the relative levels of Rac1 or RhoA activity determine which mode of motility a tumor cell uses and speculated that a tumor cell would use whichever mode provided the most efficient movement through the cellular microenvironment.

While the above reports demonstrate RhoA and its effectors play a central role in promoting adaptation to the cellular microenvironment, the regulation of RhoA in determining mobility is only beginning to be unraveled. PDK1 has been identified as a regulator of amoeboid cell motility through maintenance of ROCKI activity. PDK1 competes with RhoE for binding ROCK1 and prevents inhibition of ROCK1 by RhoE, specifically at the plasma membrane (83). Smurf1 (81), EphA2 (85), and loss of p53

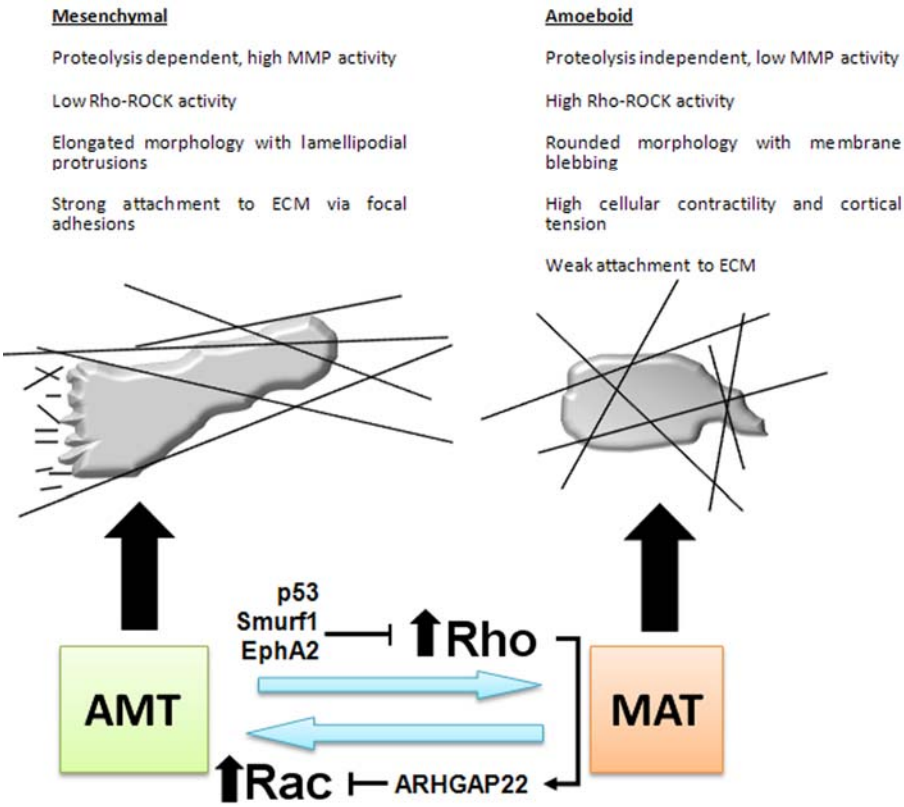

Figure 5. Rho-ROCK signaling is a pivotal regulator of tumor cell plasticity. Migrating tumor cells exhibit plasticity for the type of motility they use. Cells that typically use a mesenchymal, metalloproteinase-dependent mode can convert to an amoeboid mode (mesenchymal to amoeboid transition, MAT) when extracellular proteolytic activity is pharmacologically inhibited. Conversely, amoeboid cells switch to a mesenchymal motility (amoeboid to mesenchymal transition, AMT) when RhoA or ROCK activity is inhibited. This plasticity from mesenchymal to amoeboid and amoeboid to mesenchymal motility appears to be high dependent on an inverse relationship between Rho and Rac activity.

(86) have also been identified as positive regulators of MAT. The ability of each of these proteins to regulate amoeboid motility was, again, linked to promotion of RhoA or ROCK activity at the membrane protrusions, reinforcing the notion that RhoA and ROCK activity play an important role at the front end of migrating cells.

Lastly, alterations in Rho activity can have important pathophysiological implications for tumor cell invasion as mesenchymal to amoeboid conversions, and vice versa, are also observed *in vivo*. In agreement with the reports correlating RhoA expression with metastatic potential, conversion of cells from mesenchymal to amoeboid motility (high Rho activity) was associated with greater tendency for metastasis in mouse models (79, 82-85). Cells induced to undergo MAT (high RhoA) showed increased colonization to the lung (84, 85); while induction of AMT (low RhoA) decreased lung colonization by 60% (83).

6. PERSPECTIVE

The function of RhoA was initially probed by using inhibitors and point mutants to perturb its activity in standard 2-dimensional culture systems, which identified important roles for RhoA in generating traction and contractile forces. Models for cell migration in these systems typically limited the role of RhoA to the back end of the cells, as it was thought to negatively regulate protrusion at the front of the cell. However, studies of tumor cell invasiveness identified a strong positive correlation with RhoA were at odds with the classical viewpoints restricting RhoA action to the back end of migrating cells.

We have discussed how two paradigm shifting findings have modified tumor migration models to reconcile this discrepancy. First, the development of sophisticated FRET biosensors revealed that RhoA activity is not restricted to the back end of migrating cells, but is also present in membrane protrusions at the front end. This was an extremely unexpected finding, and established the possibility that RhoA can positively influence protrusion at the leading edge. Second, the use of 3-dimensional cultures systems revealed that tumor cells use multiple invasive mechanisms to navigate through complex environments. The recognition that malignant tumor cells often use an amoeboid style of migration driven by Rho-ROCK dependent blebbing provided a new conceptual framework for cell migration. Thus, the view of the RhoA pathway in cell invasion and metastasis has evolved to

recognize that RhoA can promote protrusive and invasive behavior of cells.

Studies since the 1990's have established that the RhoA signaling module is incorporated in the regulation of a wide variety of cellular processes, including tumor metastasis. A current challenge is to understand how the upstream and downstream regulators of RhoA signaling are modulated to coordinate complex behaviors such as invasiveness. The spatial regulation of RhoA and its effectors is one means by which signaling downstream of RhoA may be regulated. In addition, there are 60-80 Rho GEFs and GAPs, which are often multi-domain proteins that could integrate upstream signals to coordinate RhoA signaling. In this review we have discussed the first wave of experiments designed to probe the modifiers of RhoA signaling during metastasis; we anticipate the future will elucidate more nuances of RhoA regulation that will continue to evolve our understanding of how malignant cell harness cues from the microenvironment to disseminate through the body.

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