AMOEBOID TRANSITION OCCURS IN MAMMILIAN TUMOR CELLS IN RESPONSE TO CHANGES IN SPACIAL CONFINEMENT AND ADHESION

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by RICARDO REYES titled AMOEBOID TRANSITION IN MAMALLIAN TUMOR CELLS IN RESPONSE TO CHANGE IN SPACIAL CONFINEMENT AND ADHESION and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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Abstract

In this dissertation, we review how plasticity in the modes of cell migration can occur in response to changes in the extracellular matrix. Different modes of migration exhibit varying characteristics, such as cell adhesion, membrane protrusion, and proteolysis, allowing tumor cells to adapt to their current extracellular environment, thus enhancing invasive behavior in response to different antitumorigenic therapies. The combination of various cell migration characteristics results in a distinct mode of single-cell migration, which, for single-cell migration, falls under two general modes, mesenchymal-traction-force motility or amoeboid-propulsion-squeeze motility.

Mesenchymal-to-amoeboid transition is known to occur when tumor cells are in a softer matrix with low adhesion and in a high-confinement environment. Under these conditions the tumor cells exhibit higher levels of cortical myosin activity. Amoeboid migration is characterized by high cortical contractility, mediated by increased myosin activity along the cell cortex. Understanding how contractility is increased under changes in adhesion and confinement is important to uncover the possible mechanisms a tumor cell uses to optimize motility. Here, I will introduce a model that explores how the differential regulation of RhoGTPases (Rac1 and RhoA) is modulated with changes in cell-matrix adhesion and cell-matrix confinement to induce mesenchymal-to-amoeboid-transition. In the model, tumor cells in soft matrix are exposed to fewer ligands to which they can bind their integrins and activate Rac1. Loss of Rac1 activation in soft matrix inhibits lamellipodia formation, and the double-negative relationship between Rac1 and RhoA will shift towards RhoA to promote increase cortical myosin activity for amoeboid migration (Figure 1). Myosin activity is further enhanced under confinement through retrograde flow and recycling of transmembrane protein like integrin and syndecan, which results in increase of RhoA-mediated contractility for amoeboid migration.

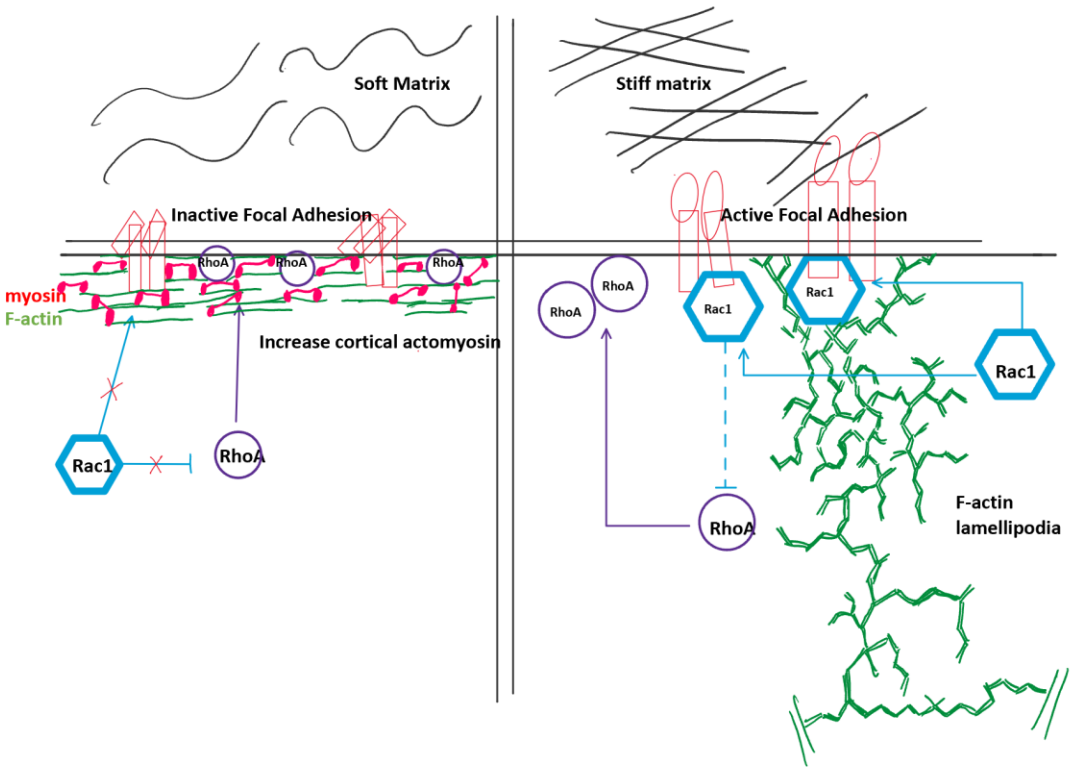

Figure 1: Left panel, Cell membrane with focal adhesion in soft matrix. There are fewer ligands in soft matrix for focal adhesions to interact, resulting in inactivation of focal adhesions. Rac1 will not translocated to focal adhesions and become active. Inactive Rac1 will prevent inhibition of RhoA. RhoA activation will promote actin and myosin formation along cortical membranes and enable amoeboid transition. Right panel, Cell membrane in stiff matrix. Focal adhesion will bind to abundant and exposed ligands to become activated. Activated focal adhesions will recruit Rac1 to activate actin polymerizing proteins for lamellipodia. Rac1 also inhibits RhoA to prevent myosin activity. Activation of Rac1 favors mesenchymal migration.

Introduction

Plasticity in the modes of migration is important in developmental biology, tumor progression, and immune activity (Friedl and Wolf, 2010). This migratory plasticity is described as a reversible transition between different modes of migratory phenotypes. There are two dominant modes a migrating-single-cell can develop, the amoeboid mode which has a round shape and squeezes its plasma membrane to move through the environment, and the other is the mesenchymal mode which is elongated and uses adhesion molecules to move. Each mode has its own distinct cytoskeletal features and adhesion properties to produce different forms of mobility

within their respective tissue environment (Figure 2). Cells in normal tissues are connected and supported by an extracellular matrix scaffold that can influence cell shape and behavior through its biochemical and mechanical stimuli (Frantz et al., 2010).

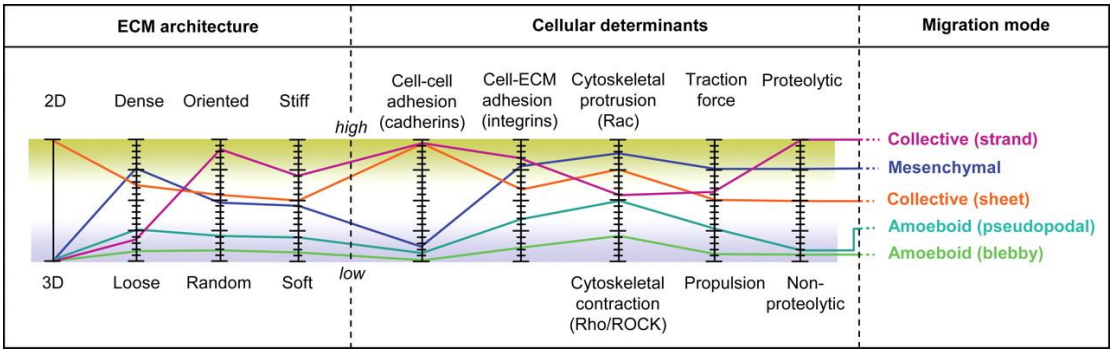

Figure 2: Cellular and ECM determinants for respective modes of cell migration. Collective migration (pink/orange) will be favored with loose and stiff ECM, high cell-cell adhesion, high cell-matrix adhesion, cellular protrusion via Rac and high proteolysis. Mesenchymal mode (dark blue) is favored in dense and stiff matrix, very low cell-cell adhesion, high cell-matrix adhesion, high cellular protrusion and proteolysis. Amoeboid (green/light blue) is favored under lose and soft ECM, low cell-cell adhesion, low cell-matrix adhesion, high cytoskeleton contraction via RhoA/ROCK, low protrusion and no proteolytic activity. Cell shape and behavior is modulated in response to ECM changes that cause changes in cellular signaling. Figure from: Friedl, P., and Wolf, K. (2010). Plasticity of cell migration: a multiscale tuning model. The Journal of Cell Biology *188*, 11-19. Creative Commons; https://creativecommons.org/licenses/by-nc-sa/3.0/

The extracellular matrix is part of a microenvironment that is highly dynamic, because it is constantly being remodeled to achieve tissue structure homeostasis. Under pathological conditions, like cancer, matrix components are dysregulated resulting in drastic changes to the extracellular environment. (Walker et al., 2018). Cancer cells can use cell plasticity to optimize survivability and invasion in the altered matrix. A similar adaption can occur in tumor cells in response to chemotherapies (Taddei et al., 2014). When an anti-tumor therapy is targeting a component of cell adhesion that is necessary for mesenchymal cell migration, the tumor cell can initiate a transition to the alternative amoeboid mode and provide chemoresistance (Taddei et al., 2014; Talkenberger et al., 2017). This tumor cell plasticity is also useful for immune-surveillance escape. When immune cells are targeting tumor mesenchymal components, the tumor cell can transition into an amoeboid mode and prevent recognition from immune cells (Lorenzo-Sanz and Muñoz, 2019; Terry et al., 2017). Although tumor cells can exhibit both types of migratory modes, those that can transition from mesenchymal to amoeboid are associated with aggressive

metastatic cancers (Pastushenko and Blanpain, 2019). Understanding the causes of the tumor cell transitions can potentially serve as a platform to develop new interventions that help prevent invasion, chemoresistance, and immune escape in cancer patients.

The transition between the two modes is correlated with aberrant changes in the extracellular matrix (like growth factor and cytokine expression), chemotaxis, durotaxis, spatial confinement, hypoxia, cell-matrix interactions, and cell-cell interactions, all of which are progressive features of tumorigenesis (Wang et al., 2017). These changes are thought to be created through genetic mutations and abnormal signaling between tumor cells and benign cells, resulting in a complex environment that enhances transitions between modes of cell migration and optimizes malignancy (Hecht et al., 2015).

Mesenchymal to amoeboid transitions can be influenced by the mechanical properties of the extracellular matrix (ECM). The mechanical signal of the matrix can be finely-tuned by altering matrix stiffness/rigidity, adhesion, and spatial confinement (Bergert et al., 2012; Friedl and Wolf, 2010; Panková et al., 2010; Talkenberger et al., 2017). In general, increasing cell-matrix adhesion (seen in stiff matrix) and decreasing cell-matrix confinement can support a mesenchymal cell phenotype (Friedl and Wolf, 2010). On the other side of the transition spectrum, by decreasing cell-matrix adhesion (seen in soft matrix) (Alvarez-Gonzalez et al., 2015; Ruprecht et al., 2015) and increasing cell-matrix confinement (Liu et al., 2015), it can promote an amoeboid cell phenotype. The new model in this paper may explain why we see tumor cells undergo these changes under different environmental conditions.

The mechanical mechanism to promote these phenotypes in their respective conditions is still unknown. However, there is a significant difference in cortical contractility when cells are in different matrix adhesion and confinement conditions. This may explain why there are transitions between cell migration, since amoeboid mode is heavily dependent on cortical contractility (Jacobelli et al., 2010; Ruprecht et al., 2015). Additionally, RhoA is a mechanical-sensitive

GTPase that is differentially up-regulated in amoeboid migration and causes an increase in myosin contractility (Parri and Chiarugi, 2010). Here, we will explore different signaling pathways that regulate RhoGTPases (RhoA and Rac1) and then speculate how they could be modulated under different cell-matrix adhesion and cell-matrix confinement to favor mesenchymal-to-amoeboid transition

Modes of Migration: Collective and Individual Cell Migration

Cell migration is facilitated through either a collective action, by traveling bound to other cells, or by being a single-cell that translocates individually to a distal site (Friedl and Alexander, 2011). Single-cell migration carries two extreme subclasses or modes that are distinguishable by their phenotype, amoeboid and mesenchymal. The difference in appearance and motility between the two single-cell modes are due to changes in membrane signaling, actin structure, myosin activity, expression of adhesion molecules, and gene expression (Figure 2) (Brábek et al., 2010). How the cells respond to the extracellular matrix (ECM) can favor the signaling programs for one of the two individual modes (Figure 2). Tumor cells can take advantage of these signaling programs and engage in different motility strategies when they encounter challenges like: ECM changes (Bergeman et al., 2016), anti-tumor therapies (Friedl and Alexander, 2011; Taddei et al., 2014), immunosurveillance (Lorenzo-Sanz and Muñoz, 2019; Terry et al., 2017), and metabolic stress (Lehmann et al., 2017).

Mesenchymal Cell Migration

One of the two single-cell modes is the mesenchymal phenotype. Mesenchymal cell migration is classified by its use of adhesion molecules and matrix metalloproteinases (MMP) (Table 1) (Wolf et al., 2003). MMP are proteolytic enzymes that cleave and degrade large building blocks of the extracellular matrix, such as collagen and fibronectin. As a result, the proteolytic enzymes can remodel the ECM into a roadway and expose adhesion ligands, allowing cells to move towards their respective chemotactic or durotactic signals (Frantz et al., 2010).

Integrins are adhesion molecules on cells that bind to ECM components, like collagen or fibronectin, to create and activate focal adhesion clusters. These focal adhesions (FA) activate downstream signaling proteins to recruit and polymerize actin filaments at the leading-edge (front end of the cell) to create long spindle membrane extensions called lamellipodia (Nobes and Hall, 1995) (Table 1; Figure 3).

	Mesenchymal migration	Amoeboid migration
Shape	Elongated, spindle shape, polarized	Round, irregular shape, non-polarized
Protrusions	Lamellipodia	Blebs
Adhesion	High adhesion	Low adhesion
Velocity	Slower	Faster
Protease	High activity	Low activity
Mobility	Traction-Force motility	Propulsion-squeeze motility

Table 1: differentiating traits between mesenchymal- and amoeboid-cell migration.

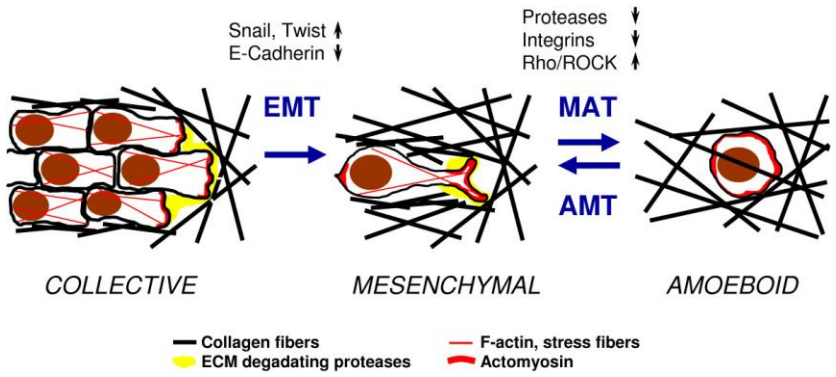

Figure 3: Modes of cell migration. Collective migration has leader cells with ability to degrade ECM for mobility. The trailing cells, which are behind the leader cells, have epithelial characteristics such as high levels of cell-cell junction E-cadherin. Epithelial-to-Mesenchymal Transition (EMT) is a process which allows collective-epithelial cells to transition into mesenchymal cells by decreasing E- cadherin junctions and increasing snail and twist transcription factors. The combination of signals allows the cell to rearrange actin and myosin to change cell shape and affect its mobility. Mesenchymal-to-Amoeboid Transition (MAT) is the transition from mesenchymal cell to amoeboid cells. Up-regulating RhoA and ROCK kinase further re-arranges actin and myosin along the membrane, around the cell to alter cellular mobility. Figure from: Brábek, J., Mierke, C.T., Rösel, D. et al. Cell Commun Signal (2010) 8: 22. Creative Commons: https://creativecommons.org/licenses/by/2.0/

Along with lamellipodia, mesenchymal cells can also create filopodia at the leading-edge of the cell. Filopodia are smaller protrusions that not only help stabilize focal contacts by weakly binding to ECM, but are also able to respond to chemokines for directed migration (Caswell and Zech, 2018). Lamellipodia and filopodia are primarily regulated by polarized the RhoGTPases, Rac1 and Cdc42, at the leading-edge of the cell as a result of new focal adhesion activation (Nobes and Hall, 1995). Consequently, focal adhesion can establish cellular polarity through

Rac1 and Cdc42 in mesenchymal cells. Since amoeboid cells cannot form mature focal adhesions, these cells will not initiate the necessary Rac1 and Cdc42 to shape and polarize the cell (Table 1; Figure 3).

Mesenchymal cells generate focal adhesions using integrins, which are membrane-bound molecules that bind to the ECM when they are in an active state (Figure 4). Active-integrin recruits adapter proteins and actin-binding proteins like talin, vinculin and paxillin (Vicente-Manzanares et al., 2009). Active Focal Adhesion Kinase (FAK) and Src Kinase then localize and bind to the adapter proteins that are bound to active adhesion molecules (Westhoff et al., 2004). With FAK and Src Kinase at the membrane, the probability to activate Rac1 and Cdc42 increases. Active Cdc42, Rac1 and P21-Activated Kinase (PAK) can activate Arp2/3 via WASP/WAVE activation (Figure 4) (Vicente-Manzanares et al., 2009). Arp2/3 is an actin-polymerizing protein that helps create the lamellipodia extension at the leading-edge, towards the direction of migration (Figure 4) (Panková et al., 2010; Vicente-Manzanares et al., 2009). At the trailing-end (the back of the cell), there is

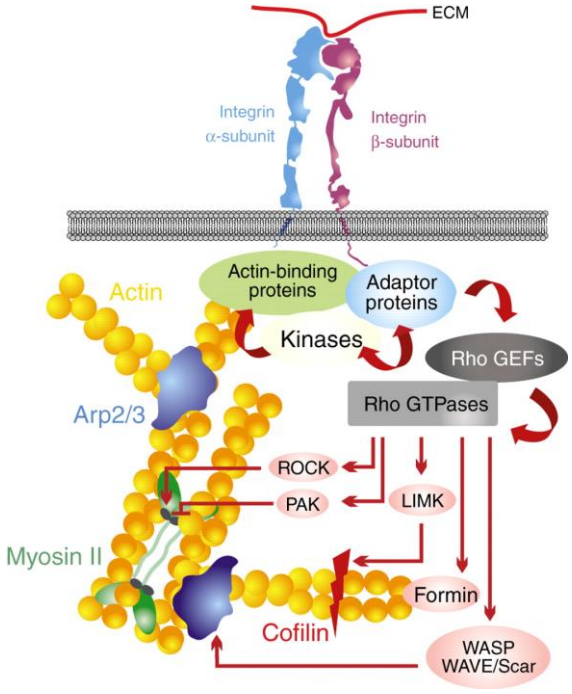

Figure 4: Many integrins bind to ECM components (collagen or fibronectin) to form focal adhesion complexes. Active integrin domains recruit actin-binding and adaptor proteins which facilitate activation of kinases such as FAK and Src. This leads to activation of downstream effector proteins like RhoGTPases and PAK to activate WAVE and Arp2/3 for actin polymerization at the focal adhesion. Figure from: Vicente-Manzanares, M., Choi, C.K., and Horwitz, A.R. (2009). Integrins in cell migration--the actin connection. Journal of Cell Science *122*, 199-206. By permission of Journal of Cell Science.

formation of actin-myosin stress fibers that mediate forward retraction of the rear. Localization of the stress fibers towards the rear is regulated by polarized RhoA activity at the trailing-end (Figure 5). Displacement of mesenchymal cells is mediated by a traction force that is created when a cell forms focal adhesions and has polarized signaling of Rac in the front of the cell and RhoA in the back of the cell to coordinate mobility (Figure 5) (Pandya et al., 2017b). As a result, mesenchymal cell migration is typically slower compared to amoeboid cell migration because it must make new focal adhesions every time the cell moves forward (Pandya et al., 2017b).

Cell movement is initiated when active focal adhesions cause the cell to anchor to the ECM and provide a point for traction-force (Figure 5). Active Rac1 and CDC42 at the leading-edge initiate formation of lamellipodia to extend the membrane past the current integrin anchor, and create multiple new focal adhesions in the direction of migration (Pandya et al., 2017b). At the trailing-end of the cell, myosin stress fibers contract and generate tension force between trailing- and leading-edges. Focal adhesions at the rear are weaker compared to the new and multiple focal adhesions at the front of the cell. Tension force from stress fiber contraction overcomes the anchor force at the rear, causing release of rear adhesion sites (Pandya et al., 2017b). Once the rear is unbound, the stress fibers then facilitate membrane retraction and

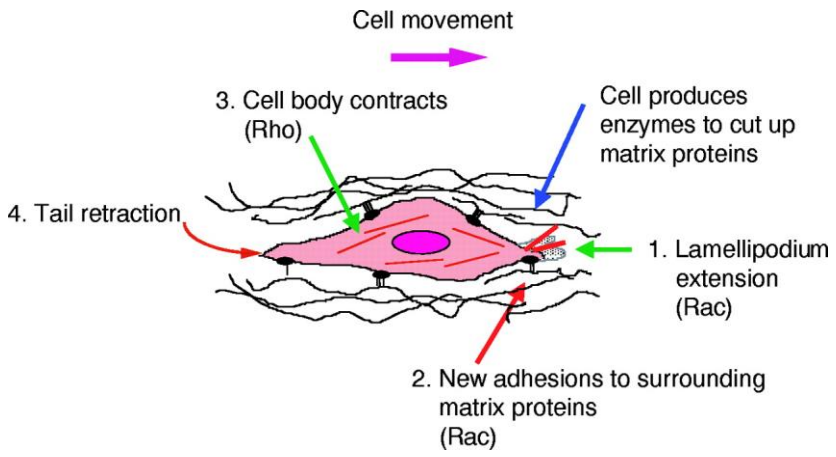

Figure 5: General mesencymal cell migration. Cells begins movement in response to a stimulus. Focal adhesion signaling initiates lamellipodia extensions in the direction of the stimulus and create focal adhesions. At the trailing-end of the cells there is increased contraction by stress fibers. Weakened rear adhesion points release and allow retraction of membrane for displacement. Ridley, A.J. (2001). Rho GTPases and cell migration. Journal of Cell Science *114*, 2713. By permission of Journal of Cell Science.

translocation of the membrane forward (Figure 5). The old anterior adhesion points now serve as the posterior traction anchor, as the cell restarts lamellipodia extensions to continue the cycle (Figure 5) (Lauffenburger and Horwitz, 1996) (Sheetz et al., 1998).

Other distinctive features associated with mesenchymal cells include: increase in expression of N-cadherin (which is another adhesion molecule that binds to the extracellular matrix), increase in expression of vimentin (a well-known intermediate filament found in mesenchymal cells), decrease in E-cadherin expression (a cell-cell adhesion molecule), and increase in secretion of proteolytic proteins (Clark and Vignjevic, 2015; Panková et al., 2010).

Amoeboid Cell Migration

The second distinguishable mode of single-cell migration is amoeboid phenotype. It is phenotypically distinct from mesenchymal migration (Figure 3; Table 1). Amoeboid mode has a rounded cell shape and multiple smaller membrane protrusions called blebs (Figure 6). The migration is independent from any adhesion or proteolysis, which is the opposite of mesenchymal migration (Wolf et al., 2003). The differences in membrane protrusions and independence from ECM adhesions are a result of having a different actin-myosin organization (Byrne et al., 2016)

The amoeboid cell phenotype is strongly correlated with higher levels of RhoA, which increase contraction of stress fibers throughout the cell. The contractions increase the intracellular pressure in the cell, which disrupt the actin along the cortical membrane (Figure 6) (Fackler and Grosse, 2008). Force generated from the pressure pushes the plasma membrane that has been detached from the actin cytoskeleton, creating blebbing (irregular-shaped) protrusion (Figure 6). The bleb shape is dependent on the degree of pressure created by actin-myosin activity (Pandya et al., 2017a, b). After the membrane is extended, there is recruitment of Ezrin-Radixin-Moesin (ERM) family proteins to the intracellular side of the membrane bilayer. ERM proteins help recruit RhoA and ROCK to the membrane to localize myosin (Fackler and Grosse, 2008).

Concentrated actin and myosin initiate contraction and retract the bleb back to its starting point (Figure 6) (Fackler and Grosse, 2008).

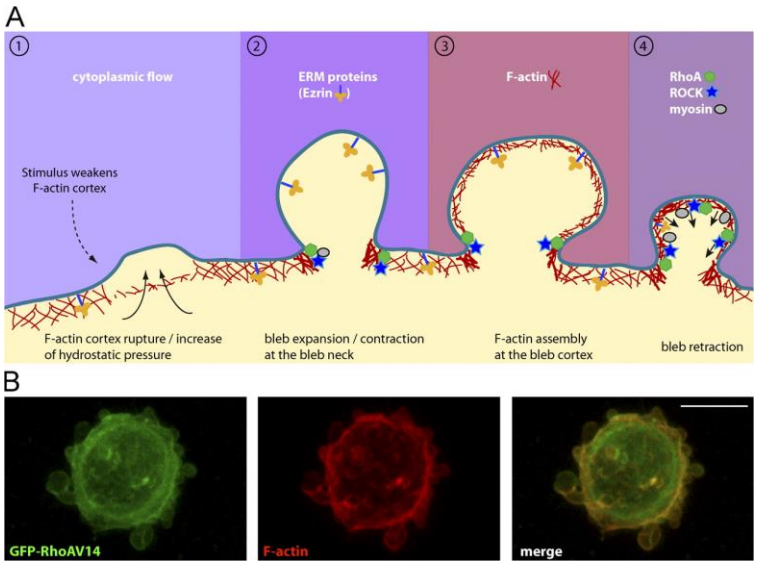

Figure 6: A: Actomyosin dynamics in bleb formations. Increased hydroststaic presssure from rearward actomyosin contraction causes F-actin at the cortex to rupture. Plasma membrane is extended from pressure causing blebbing extensions. ERM proteins on the exposed plasma membrane recruit RhoA for myosin and actin reogranization along the membrane. New actomyosin along the bleb cortex retracts the plasma membrane back to the cell. Cylce continues to make consistent contacts along the matrix to facilitate displacement B: GFP-RhoA epxression is high along cortical membrane. F-actin red is highly expressed on cortical membrane. Colocalization of green RhoA and red F-actin is located at cortical membrae. Figure from Fackler, O.T., and Grosse, R. (2008). Cell motility through plasma membrane blebbing. The Journal of Cell Biology *181*, 879. By permission Journal of Cell Biology.

When the cells are not able to form focal adhesion, displacement of amoeboid cell migration is achieved through retrograde flow of the plasma membrane, actin filaments, and myosin (O'Neill et al., 2018). Retrograde flow is maintained through endocytic lipid vesicles and driven by an actin-myosin contraction gradient (Figure 7) (Moreau et al., 2018; O'Neill et al., 2018). Contractile stress from concentrated myosin at the rear and cortical membrane will cause actin filaments to break into individual protein monomers. Individual actin proteins then flow to the front of the cell to be added to the growing end of the actin structures, resulting in a retrograde flow of actin. Myosin will follow actin to continue making contractile units with actin, and together will move to the membrane (O'Neill et al., 2018). As the actin polymerizes, it generates very small membrane ruffle-protrusions that can generate frictional force between bilayer and

extracellular matrix structures (Figure 7) (O'Neill et al., 2018). The non-specific frictional force causes a squeezing motion, resulting in cell displacement (Fackler and Grosse, 2008; Moreau et al., 2018).

Interestingly, it is not just the membrane that moves during retrograde flow of actin, but also transmembrane proteins are flowing rearward (O'Neill et al., 2018). Fluorescent beads attached to the membrane bilayer were seen to be flowing and pushing rearward, towards an optically-stimulated RhoA, confirming the membrane flow and control by RhoA-myosin contractility (O'Neill et al., 2018). Labeled transmembrane proteins, like integrins or GPCR, and intracellular signaling lipids, like PIP2 or farnesyl lipid, are also flowing along with the membrane (Hawkins et al., 2011; O'Neill et al., 2018). As a result, there is an accumulation of important cortical actin proteins such as ezrin, moesin, and intracellular signaling lipids like PIP2, which can maintain or enhance RhoA signaling (O'Neill et al., 2018).

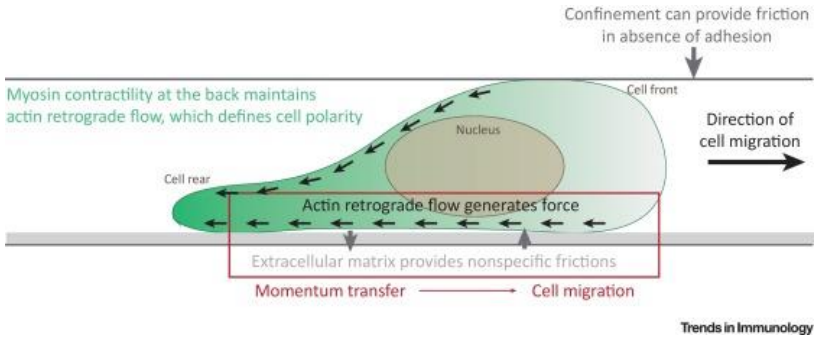

Figure 7: When cells are placed on ECM in the absence of adhesion, cells can create frictional forces between cell membrane and ECM molecules. Force is genertaed by continuous retrograde flow of actin. Retrograde flow is maintained by a polarized gradient of myosin and RhoA activity to apply stress on rear-actin filaments. Polyermization of actin causes force to be applied on membranes, the momentum force from protrusion is transferred to ECM molecules causing the membrane to propel against the surface resulting in cell migration. Frinctional force is enhanced when cells are in confinement plus low adhesion enviroments. The rate of retrograde flow increases to account for the increased fictional forces in confinement. Figure From Moreau, H.D., Piel, M., Voituriez, R., and Lennon-Duménil, A.-M. (2018). Integrating Physical and Molecular Insights on Immune Cell Migration. Trends in Immunology *39*, 632-643. By permission of Elsevier.

Mobility can also be generated within viscous fluids exhibiting a swimming motility that is similar to the squeezing motility (O'Neill et al., 2018). The frictional forces from the membrane retrograde flow allow the cell to move in a low adhesion environment, a highly confined

environment, and even in a viscous-fluid environment (Moreau et al., 2018; O'Neill et al., 2018). When amoeboid cells are placed in a confined or spatially restrictive environment (like small openings in the matrix), the frictional force is increased due to increased contact points along the cell membrane, which enhance motility even under very non-adhesive conditions. This mechanism is called “chimneying” (Moreau et al., 2018). Chimneying may explain why amoeboid cells are faster and favored under highly-confined matrix environments (Ruprecht et al., 2015).

Once the cells are mobile, the directional cues can be interpreted differently between amoeboid and mesenchymal cells. Key mediators in directionality are durotaxis (movement along a stiffness gradient), chemotaxis (movement along a chemical gradient), and haptotaxis (movement along an adhesive-substrate gradient) (Bear and Haugh, 2014).

Nuclear deformation is a large determinant in durotaxis. It can dictate the direction in which amoeboid cells move in a 3-dimentional (3D) matrix. 3D matrices contain many holes/pores with various sizes. A matrix with higher amounts of smaller size holes/pores has a rigid/stiff matrix made from higher concentration of ECM components, like collagen or fibronectin (Walker et al., 2018). In contrast, a softer matrix with larger holes/pores has a less rigid matrix made from less concentrated collagen or fibronectin (Walker et al., 2018). Amoeboid cells prefer to move onto areas of the matrix with larger-size holes (less rigid) to avoid nuclear stress/deformation, because it is not able to degrade the stiff matrix using proteases (Renkawitz et al., 2019). Amoeboid cells can select and direct their migration by using their nucleus as a gauge to assess pore size within the ECM and preferentially move towards loose-open matrix spaces. (Friedl et al., 2011; Renkawitz et al., 2019). The nucleus is a very large organelle, and it limits where the cell can move if it does not have a means to make the space larger. In contrast, when passing through smaller holes, mesenchymal cells can reposition their nucleus towards the back of the cell and secrete proteolytic enzymes at the leading-edge of the cell to make the pore size

larger (Friedl et al., 2011). Proteolysis of the ECM will also expose ECM ligands for the cell to create focal adhesions. Mesenchymal cells will prefer and be directed towards more rigid/stiff ECM, since it is able to increase ECM ligands to make focal adhesion and begin traction motility (Wolf et al., 2003). The two migration modes respond differently when exposed to the same durotactic signal because of their distinct perinuclear dynamics, ability to form adhesion, and ability to secrete proteases.

Haptotactic cues strongly influence mesenchymal cells, because of their dependence on adhesion molecules to drive motility. Mesenchymal cells move in the direction of high concentration of adhesion molecules, which differ from amoeboid cells that move independent of focal adhesion (Wolf et al., 2003). Durotaxis can be associated with haptotaxis, because adhesion molecules tend to be concentrated in very rigid/stiff matrix (Frantz et al., 2010; Wolf et al., 2003).

Amoeboid cells under a chemokine gradient can respond similarly to mesenchymal cells, especially if the cell type is the same. However, when looking at distinct cell types, such as typical mesenchymal fibroblasts and amoeboid lymphocytes, their chemotactic sensing is mediated by different types of chemoattractant receptors (Bear and Haugh, 2014). Generally, amoeboid lymphocytes use G-Protein Coupled Receptor (GPCR) to sense interleukin or chemokine ligands for directional migration. Mesenchymal fibroblasts can express Receptor Tyrosine Kinase (RTK) receptors to sense their respective growth factors and drive migration towards the chemical signal (Bear and Haugh, 2014). Even though the downstream signaling of these receptors are intertwined, the regulation and inhibition of each is substantially different (Bear and Haugh, 2014), thus adding to the list of differential features between the two modes.

Collective Cell Migration

Aside from the two single-cell modes of migration, cells can move collectively while adhered with others in a cluster or in a stream of cells (Figure 3). A large determinant of this process is the ability to express junctional proteins, such as E-cadherin (cell-cell adhesion

molecule), which cause cells to remain stuck to each other as they move in the environment (Clark and Vignjevic, 2015). The collective group of cells has a leader cell that exhibits the properties of single-cell mesenchymal migration. The follower cells display an epithelial phenotype with high levels of E-cadherin (cell-cell junction), and has a basal-apical cell polarity (Pandya et al., 2017a). The leader cell is also interconnected with the follower cells through cell-cell junction and an actin-myosin network (Fernandez-Gonzalez et al., 2009). As the leader cell moves forward, leader cells secrete proteases that degrade the ECM and create micro-tracks, which allow the epithelial cells to drag along (Figure 3) (Haeger et al., 2014; Pandya et al., 2017a).

Although the regulation of collective migration is more complex than a single-cell mesenchymal, they still share features, such as having a traction-mediated migration via focal adhesions (Pandya et al., 2017a). Leader cells project lamellipodial protrusions to create focal adhesions, and the lagging-epithelial end undergoes RhoA-ROCK-mediated contraction (Pandya et al., 2017a). Contraction is further enhanced by having the lagging-epithelial cells interconnected with other cells via cell-cell junctions, which allow synchronous contraction and retraction (Fernandez-Gonzalez et al., 2009). Coordinating lagging-end contraction with leading cells' focal adhesions enables large-scale traction and retraction that is similarly seen in single-cell mesenchymal migration (Figure 5) (Fernandez-Gonzalez et al., 2009; Friedl and Alexander, 2011).

Each cell mode has a differential response to a durotactic, heptotactic, and chemotactic signaling. This suggests that cells can transition between various modes of migration. Tumor cells can activate various signaling programs to create specific cell behavior and morphology when they are placed in a complex and changing extracellular matrix.

Changing the Extracellular Matrix

Having a complex tumor environment that is composed of abnormal chemical and structural components can result in severe remodeling of the ECM. This leads to a new exposure of environmental cues for tumor cells and benign cells, which will affect the plasticity of cell migration (Brábek et al., 2010). The ECM acts as a physical scaffold and provides biochemicals cues to influence the gene expression of surrounding cells. The ECM composition is specific to tissue type and varies in biochemical and biophysical signaling. For example, in more soft-compliant tissues, such as breast, prostate, and lung tissue, the matrix predominately has type I and III collagen with sparse fibronectin throughout, resulting in a high resistance against stretching forces (Alford et al., 2015). In stiffer matrices like bone tissue, the ECM is produced by osteoblasts secreting large amounts of type I collagen and inorganic minerals like hydroxyapatite, providing most of the bone mass. This reduces the matrix elastic capabilities but improves compressive resistance (Alford et al., 2015). In order to maintain homeostasis in soft or hard ECM, there are specialized cells called fibroblasts within the ECM that secrete metalloproteases (MMP), crosslinking proteins (such as LOX), and the respective inhibitors for the proteases and crosslinking proteins when they are needed (Darby et al., 2014; Frantz et al., 2010).

In pathological states such as chronic inflammation, wound healing, and tumorigenesis, the ECM homeostasis is compromised as a result of over-activated fibroblasts or osteoblasts. These remodeling cells cause drastic changes to the ECM leading to a softer or stiffer ECM, ultimately affecting ECM adhesive and spatial confinement properties (Brábek et al., 2010; Darby et al., 2014). The exposure of new mechanical signaling (such as confinement and adhesion) in the new pathogenic ECM will influence the migratory and behavior traits of stromal cells and tumor cells.

In tumors, a major player in remodeling the matrix is the population of cancer-associated fibroblasts (CAFs), also classified as myofibroblasts. CAFs are fibroblasts that have been overactivated by either tumor-secreted growth factors, physical stress from a stiffer matrix, and cell-cell signaling with a tumor cell (Wang et al., 2017). Normal fibroblasts are active in inflammatory process, wound healing, and tissue matrix remodeling (Grotendorst et al., 2004). Once fibroblasts complete their function during wound healing, they become inactive and undergo apoptosis (Darby et al., 2014). However, fibroblasts in a tumor environment are exposed to persistent signaling cues, which allow the cells to become constitutively active. Overactive fibroblasts are also seen in chronic inflammatory and in fibrotic diseases (Darby et al., 2014). With an increased population of CAFs in tumor environments, there is an overwhelming deposition of collagen and crosslinked proteins resulting in completely new and foreign environment (Wang et al., 2017). Topography of the matrix can also be affected by CAFs. These fibroblasts are able to physically contract collagen fibers together to change the arrangement of the ECM (Pakshir et al., 2019).

In vivo and *in vitro* studies, and mathematical modelling, have shown that the composition of the ECM network has a profound effect on the mode of migration in neoplastic and benign cells. The combination of cell polarization, adhesion, contractility, and protease function, will dictate the appropriate mode of migration (Panková et al., 2010; Talkenberger et al., 2017; te Boekhorst et al., 2016). The possible mechanical signaling mechanisms that control these combinations are still not fully understood. A possible explanation could be found in the differential regulation of RhoGTPases in ECMs of different composition and biophysical signaling.

EMT and MAT: A Response to Changes in ECM

Cells can sense their environment through chemical signals such as chemotactic gradients, growth factors, and lipid mediators. Binding of these chemical signals to appropriate surface or intracellular receptors cause systematic activation of proteins that are directly associated with the cytoskeleton and/or gene expression. As a result, such signals can change cellular adhesion and contractility in relation to a stimulus (Totaro et al., 2018). ECM structural components can transduce a mechanical signal by binding to surface receptors and/or directly apply membrane tension to also modulate gene expression or activity of cytoskeletal elements. The physical structures in the ECM and chemical signals will play a role in regulating the transition between modes of cell migration (Dupont et al., 2011).

Epithelial-mesenchymal transition (EMT) is a dynamic transitory process between epithelial and mesenchymal cell phenotypes. EMT is an oscillating process with many intermediate stages between the two modes. Many studies have observed this EMT oscillation to be a part of tumor dissemination, especially with breast cancers cells (Bidard et al., 2008; Hüsemann et al., 2008; Nieto et al., 2016). EMT is initiated by several signaling factors that activate a set of transcription factors (TF) that control cell matrix-adhesion molecules, cytoskeleton networks, cell-cell adhesion and cell polarization (Figure 3). Major transcription factors that promote EMT include Snail, Zeb, and Twist (Brábek et al., 2010). Snail and Twist decrease the epithelial cell-cell junctions (E-cadherin) to favor single-cell mesenchymal migration (Brábek et al., 2010). Other transcriptional regulation for EMT includes miRNA and post-translational modification (Peixoto et al., 2019).

EMT is associated with aggressive metastatic breast cancer because the process allows benign-immobile tumor cells to transition into mobile-mesenchymal cells. By increasing expression of mesenchymal factors like N-cadherin (for cell-matrix adhesion) during EMT, it allow cells to adopt a traction-force motility (Figure 8) (Nieto et al., 2016). There is also similar

co-expression of epithelial- and mesenchymal-markers in disseminating-circulatory tumor cells (tumor cells in the vasculature) suggesting that these cells are undergoing EMT while metastasizing (Gupta and Maitra, 2016; Pastushenko and Blanpain, 2019) (Nieto et al., 2016).

Focusing on mechanotransduction, breast cancer cell lines can increase EMT phenotypes through two distinct pathways, Twist1-G3BP2 and Yap/Taz pathways. These pathways use two different transcription factors that promote EMT when cells are placed in a stiff matrix environment (Totaro et al., 2018). If epithelial cells are placed in a dense and stiff environment, it causes the cells to stretch and elongate resulting in an increase in actin tension in the cell. The actin tension causes the Yap/Taz transcription factor to activate and localize to the nucleus to activate mesenchymal genes (Dupont et al., 2011; Pastushenko and Blanpain, 2019). Similarly, dense and stiff matrices have a higher concentration of substrate adhesion that result in higher amounts of focal adhesion signaling. Higher focal adhesion signaling causes an increase of integrin-dependent phosphorylation of G3BP2, a membrane anchor protein (Wei et al., 2015). Phosphorylation of G3BP2 causes a change in its structure and releases the Twist transcription factor from its regulatory domain. Release from G3BP2 membrane anchor, allows Twist to localize to the nucleus to activate mesenchymal genes like N-cadherin and suppression of epithelial E-cadherin for EMT (Wei et al., 2015).

In tumor cells, mesenchymal-to-amoeboid transition (MAT) is thought to be a subsequent step from EMT, but epithelial-to-amoeboid transition can also be seen (Figure 8) (Friedl and Wolf, 2010). The regulation of MAT is mediated by growth factors, cytokines, cell-ECM interactions and chemokines (Friedl and Wolf, 2010). Generally, if a signal can change a cell's proteolytic activity or cell-ECM adhesion abilities, it can result in MAT or AMT (amoeboid-to-mesenchymal transition) (Figure 8) (Matsuoka and Yashiro, 2014). A tumor cell experiences these change in signals when they are exposed to foreign ECM conditions. Tumor cells that are transferred to a stiffer/dense matrix are exposed to high amounts of adhesion ligands which cause

integrin signaling to increase and favors expression of proteolytic enzymes via Rac1 activation (Wolf et al., 2003). Tumor cells in a softer and loose matrix have lower adhesion, resulting in higher levels of RhoA and cortical actomyosin organization to promote a round amoeboid phenotype. Therefore, MAT can be initiated by increasing RhoA and decreasing Rac1 to favor amoeboid phenotype (Figure 8). Increasing spatial confinement has also been shown to increase cortical contractility to enhance amoeboid behavior in low adhesion conditions (Figure 9) (Liu et al., 2015). The response of increased contractility, in low adhesion and high confinement, is too rapid to be linked to a transcriptional program, and cells are not adhered to a substrate, which rules-out any active adhesion signaling programs (Liu et al., 2015). The mechanical mechanism that promote this increase in cortical contractility is still unknown. Direct changes to the cell membrane or loss of adhesion signaling, in high confinement and low adhesion, may cause a change in RhoGTPases to increase cortical contractility and support amoeboid cell migration for MAT.

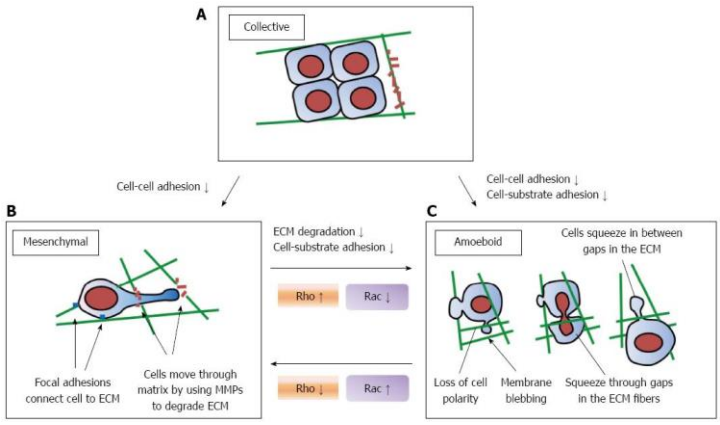

Figure 8: Transition from collective or epithelial cell migration into mesenchymal migration is caused by changes to transcriptional program mediated by Snail and Twist, which leads to a decrease in cell-cell junctions. Mesenchymal to amoeboid transition is facilitated by decreased expression of proteases, integrin adhesion and increase actomyosin contractility via Rho/ROCK induction and decreased Rac. The Rho/ROCK signaling also reorganizes the f-actin cytoskeleton around the membrane. Differential activity and expression of these proteins results in unique cell shape and behavior. Mesenchymal cells display an elongated shape with integrin at leading-edge and actomyosin at trailing-end. Amoeboid cells display a round shape with increased cortical actomyosin structures and do not have integrin adhesions. Figure from: Rho/ROCK signaling in motility and metastasis of gastric cancer. World Journal of Gastroenterology 20, 13756-13766. Permission by Creative Commons; https://creativecommons.org/licenses/by-nc/4.0/

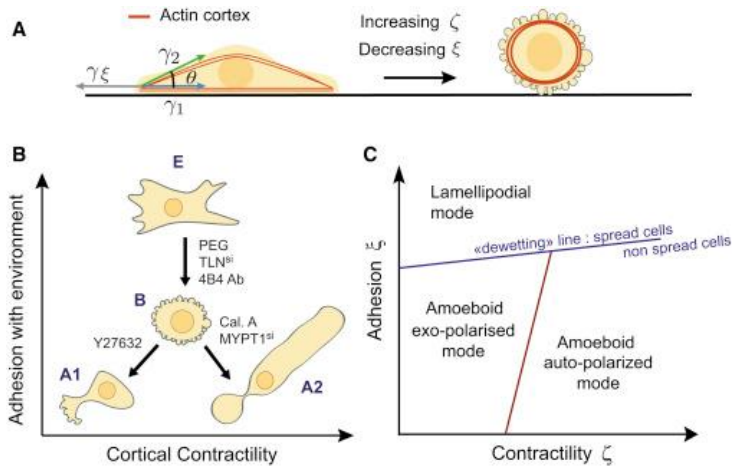

Figure 9: A: Increase in contractility of a cell, induce by confinment, and decreasing adhesion, promotes mesenchymal transition into amoeboid mode of migration. B,C Increased adhesion past a certain threshold promotes mesenchymal mode regardless of contractility. Decreasing adhesion favors amoeboid mode but the shape of amoeboid cells is dependent on the level contractility which can be changed by spatial confinement. Liu, Y.-J., Le Berre, M., Lautenschlaeger, F., Maiuri, P., Callan-Jones, A., Heuzé, M., Takaki, T., Voituriez, R., and Piel, M. (2015). Confinement and Low Adhesion Induce Fast Amoeboid Migration of Slow Mesenchymal Cells. Cell *160*, 659-672. Permission by Elsevier.

Aside from tumor cells, macrophages are also able to transition between mesenchymal and amoeboid migration in response to changes in the ECM (McWhorter et al., 2013). Macrophages can differentially exhibit M1 (pro-inflammatory, round, flat, less polarized) vs M2 (pro-healing, elongated, spindle, polarized) phenotypes, when exposed to different ECM environments (McWhorter et al., 2013). Macrophages in a dense collage matrix, with very small pores, take on a mesenchymal/M2 phenotype and increases protease secretion to break down collagen (McWhorter et al., 2013). At low-density collagen, with large size holes, macrophages adopt the amoeboid/M1 phenotype. Like tumor cells, there is a rearrangement of cytoskeleton proteins and a change in gene expression to exhibit the different phenotypes, solely through a change in the cell-ECM interaction (Pakshir et al., 2019; Čermák et al., 2018). Macrophage behavior and cell shape changes are dependent on actomyosin organization and level of contractility, both of which are controlled by the RhoA-ROCK signaling pathway (Parri and Chiarugi, 2010). During inhibition of myosin, RhoA, and ROCK, there is a loss of polarization of

the M2 and M1 phenotypes, when cultured in varying matrix stiffness as described above (McWhorter et al., 2013). The RhoA-ROCK pathway must be involved in the transitory process between mesenchymal and amoeboid phenotypes in tumor cells and macrophages.

Amoeboid migration is predominately seen in highly metastatic melanoma and breast cancer cell lines (Taddei et al., 2014). Circulating breast cancer cells in blood vessels also exhibit an amoeboid migration (Holle et al., 2019). Due to its flexible membrane and independence from cell-ECM adhesion, MAT is favorable for metastasis. Amoeboid cells can squeeze through tight spaces such as endothelial junctions (Reid et al., 2017) to aid in intravasation, mobility in fluids and extravasation from blood vessels (Holle et al., 2019). Once in circulation, amoeboid migration is also significantly faster compared to mesenchymal cell migration, which can play a factor survivability (O'Neill et al., 2018). It is reasonable to conclude that in some cell types, the MAT plays a large role in initiating metastasis, making it necessary to further understand how this process is enhanced or reduced under different ECM conditions such as stiffness, adhesion and confinement. Changes to RhoA activity are seen to drive mesenchymal cells to transition into ameoboid. Next, we will explore how regulation of RhoA is affected when tumor cells are placed in a low adhesion environment.

Bistable Signaling between RhoA and Rac1: Controlling Mesenchymal-Amoeboid Transition in Low Adhesion.

RhoA and Rac1 can control the transition between modes of migration, but first we will review the function and regulation of RhoGTPases. GTPases are molecular switches that control the activity of biochemical pathways. They are found in two conformations, an off-state and an on-state. The protein conformation is regulated by the binding of GTP (Bar-Sagi and Hall, 2000). When GTP binds to a GTPase it causes a switch to an active conformation, allowing interactions with different effector proteins to create a cellular response (Rush, 1995). When GDP is bound to the GTPase, it is in an inactive conformation and prevents the interaction with effectors proteins. Although GTPases have the intrinsic ability to catalyzes GTP into GDP, it is very slow and needs the help of an additional enzyme to regulate the GTPase activity (Rush, 1995). GTPase-activating proteins (GAPs) bind to the GTPase and enhance the catalysis of GTP to GDP to deactivate the GTPase protein when its activity is not needed (Figure 10). Guanine nucleotide exchange factors (GEFs) can switch GDP with GTP to activate the GTPase in response to a stimulus and cause a response (Figure 10) (Rush, 1995).

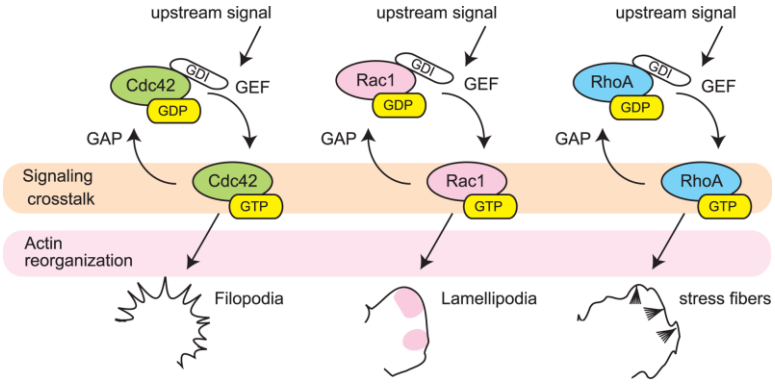

Figure 10: RhoGTPases family members (Cdc42, Rac1, RhoA) are swtiches that use GTP to change confomrational states. On-state is when GTP is bound to protein. GEF facillitates the exhange of GDP for GTP to activate the GTPase. GAP will enhance hydrolysis of GTP to GDP to inactivate the GTPase. Each GTPase has a specilized signaling function which is initiated by interacting with other proteins when GTPase is active. Cdc42 controls filopodia, Rac1 controls lamellipodia, and RhoA controls actomyosin stress fibers. Tsukada Y, Aoki K, Nakamura T, Sakumura Y, Matsuda M, Ishii S (2008). PLoS Comput Biol 4(11); Permission by Creative Commons; https://creativecommons.org/licenses/by/4.0/

Each member of the RhoGTPases family will have a different effect on cell actin organization: Cdc42 will create actin filopodia when it is active, Rac1 will produce lamellipodia, and RhoA controls actomyosin stress fibers (Tsukada et al., 2008). RhoGTPases have a diverse range of chemical and mechanical signaling that can modulate their activity. Rac1 and RhoA are the main regulators of cellular contractility and membrane protrusion (Byrne et al., 2016; Huang et al., 2014; Nobes and Hall, 1995; Parri and Chiarugi, 2010). As a result, MAT is strongly affected by the balance between RhoA or Rac1 GTPases (Figure 11).

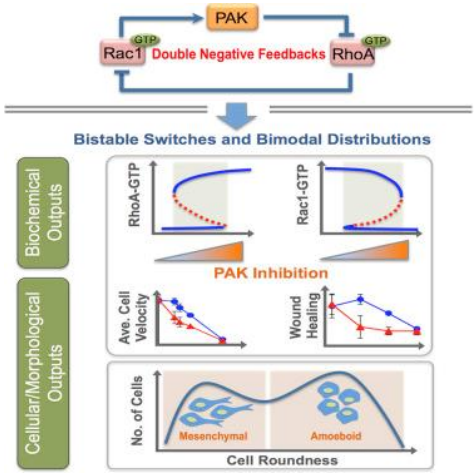

Figure 11: Double-negative feedback of Rho and Rac1 via PAK signaling. Biochecmical inputs like focal adhesion will activate PAK and increase Rac1 singaling and decrease RhoA by PAK inhibition on RhoA, promoting mesenchymal migration. If PAK is inhibited, RhoA is increased, from lack of inhibition, and RhoA will inhibit Rac1via RacGAP and pomote amoeboid migration. Figure from: Byrne, Kate M., Monsefi, N., Dawson, John C., Degasperi, A., Bukowski-Wills, J.-C., Volinsky, N., Dobrzyński, M., Birtwistle, Marc R., Tsyganov, Mikhail A., Kiyatkin, A., et al. (2016). Bistability in the Rac1, PAK, and RhoA Signaling Network Drives Actin Cytoskeleton Dynamics and Cell Motility Switches. Cell Systems 2, 38-48. Permission by creative commons; https://creativecommons.org/licenses/by/4.0/

RhoA and Rac1 have a bistable relationship through a double-negative feedback. A double-negative feedback is when two molecules have a negative interaction with each other, and if one is overactive it will decrease the negative effect from the second molecule (Figure 11). RhoA and Rac1 activity can be controlled by either cell-ECM adhesion (Carragher et al., 2006), chemical signaling by growth factors (Huang et al., 2014), cell-polarizing signals (Gandalovičová et al., 2016), and Rho-GAPs and -GEFs (Sanz-Moreno et al., 2008), all of which are dysregulated within a tumor microenvironment (Clark and Vignjevic, 2015).

Changes in ECM adhesion can cause a shift in the RhoA/Rac1 bistable double-negative network towards one extreme, facilitating the transition between modes of migration (Figure 11). For example, tumor cells with overactivation of RhoA increase contractility levels and rearrange the actin cytoskeleton to be concentrated along cortical membranes, creating an amoeboid round-shaped cell (Ruprecht et al., 2015). In contrast, cells with higher Rac1activity, favor actin polymerization at leading-edges, creating lamellipodia and increased adhesion complexes for mesenchymal migration (Huang et al., 2014). Biochemically, Rac1 and RhoA are antagonistic to each other and are even found to be polarized at opposite ends of the cell (Nobes and Hall, 1995; Tsukada et al., 2008). Rac1 is anterior and concentrated toward the direction of movement, while RhoA is found posterior to the leading-edge. Distinct polarization of RhoA and Rac1 is more apparent during mesenchymal migration, while amoeboid cells have overwhelming RhoA throughout the cells and low levels of activated Rac1(Tsukada et al., 2008). This suggests that RhoA upstream signaling and its downstream functions have a very important role in regulating the transition to the amoeboid phenotype, further supporting our model (Figure 1).

The double negative relationship between RhoA and Rac1, as described above, is linked to the p21-activated kinase (PAK), which is highly dependent on focal adhesions (Byrne et al., 2016). As the cell experiences changes to the ECM, it affects the stimulus for PAK activation and ultimately decides which RhoGTPase is favored by being able to control the double-negative network (Figure 11) (Byrne et al., 2016). Cells that form focal adhesion activate Rac1 to phosphorylate and activate PAK. Active PAK is now able to inhibit RhoA and remove the inhibition on Rac1 (Figure 11). Inhibiting RhoA further enhances Rac1 and PAK activation to maintain and overactivated Rac1 signaling, as seen in mesenchymal migration (Parri and Chiarugi, 2010). If a tumor cell is in a low adhesion space, where it is not able to form focal adhesions (like in soft matrix), then perhaps PAK will not be activated and will not inhibit RhoA signaling (Itakura et al., 2013). RhoA is released from its inhibition, allowing it to interact with

Rac1 to reduce the activity (Figure 11) (Parri and Chiarugi, 2010). Overall there are higher levels of RhoA in low cell-ECM adhesion environments which promote an increase of cortical myosin for amoeboid migration (Figure 1) (Byrne et al., 2016). FAK (Arriagada et al., 2019), Src kinase (Westhoff et al., 2004) and Yap/Taz (Nardone et al., 2017) are similar signaling components, like PAK, that are dependent on focal adhesion and influences Rac1/ RhoA double-negative mechanism. The MAT seen during low stiffness/low adhesion matrix could be explained by diminished PAK activity as a result of the lack of focal adhesion signaling. In soft matrix, there is less ligand exposed for integrins to bind and activate downstream components like PAK, FAK, Src and Yap/Taz, which could result in decreased Rac1 and increased RhoA for cortical actomyosin favoring amoeboid phenotype, which is consistent with our low adhesion model (Figure 1) (Bergert et al., 2012; Byrne et al., 2016; Friedl and Alexander, 2011; Liu et al., 2015).

Experimental manipulation (optogenetic control) of Rac1 GAPs and GEFs has shown them to be integral in establishing a spatial gradient of Rac1 and RhoA in cells to maintain mesenchymal and amoeboid migration (de Beco et al., 2018). DOCK3 is a GEF that is able to affect the RhoA/Rac1 signaling module (Sanz-Moreno et al., 2008). DOCK3 is localized at protruding ends of a mesenchymal cell to activate and maintain Rac1 (Sanz-Moreno, 2012). The DOCK3-Rac1 pathway can increase WAVE2 to decrease myosin activity and suppress an amoeboid phenotype (Figure 12) (Sanz-Moreno, 2012; Sanz-Moreno et al., 2008). Tumor cells in a stiff matrix form many focal adhesion complexes that recruit NEDD9, an adaptor protein, to form a complex with DOCK3. The complex is then able to transduce integrin signaling into Rac1 activation (Figure 12). The NEDD9 and DOCK3 complex can also enhance integrin signaling to activate more Src and FAK which further inhibits RhoA and maintains a mesenchymal phenotype (Figure 12) (Jones et al., 2017; Sanz-Moreno et al., 2008). Loss of focal adhesion (in softer matrix) results in a loss of the DOCK3 and NEDD9 complex, which leads to decreased Rac1-

mediated inhibition of RhoA. Active RhoA is now able to increase cortical contractility (Figure 12) (Sanz-Moreno et al., 2008). Downregulation of Rac1 is also mediated by activating ArhGAP22 (a Rac1-GAP), preventing the negative regulation on to RhoA/ROCK and contractility in the cell (Sanz-Moreno et al., 2008). Regulation of Rho-GAPs and GEFs during changes in cell-ECM adhesion, demonstrate the importance of Rho/ Rac1 in controlling mesenchymal-amoeboid transition.

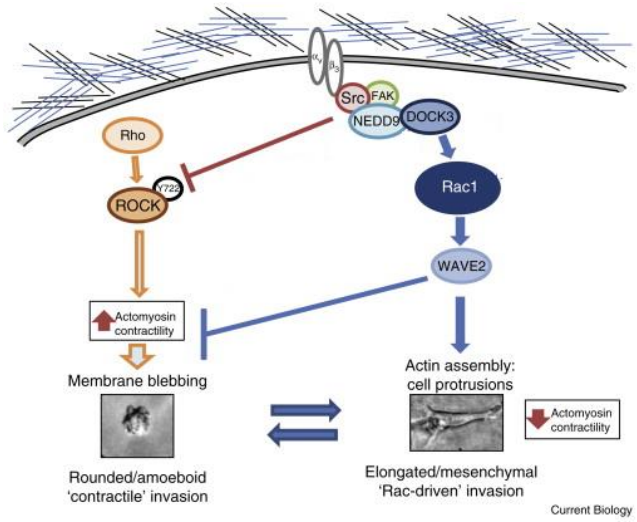

Figure 12: Similarly to PAK, NEDD9 can regulate the RhoA and Rac1 balance. NEDD9 is an adapter protein that is active with mature focal adhesions. Once active it can bind and activate DOCK3 (GEF for Rac). Rac GEF will activate Rac1 and drive tractional motility via lamellipodia. NEDD9 can also recruit Src and FAK to the integrin so they can be activated. Src and FAK will act on ROCK to inhibit actomyosin contractility to favor mesenchymal migration. If cells are placed in softer matrix, they will not be able to induce NEDD9 activation due to a lack of focal adhesions, resulting in RhoA-ROCK activation and contacility which promotes the amoeboid mode. Sanz-Moreno, V. (2012). Tumour Invasion: A New Twist on Rac-Driven Mesenchymal Migration. Current Biology 22, R449-R451. Modified. Permission by Elsevier.

Recycling and degradation of Rac1 and RhoA can regulate signaling polarization and therefore control migratory phenotype (Gandalovičová et al., 2016). Clathrin- and Rab5-mediated endocytosis is able to control the activation of Rac1 in a cell (Palamidessi et al., 2008). Clathrin is a protein that coats and forms membrane vesicles. Rab5 is a protein that sorts endosome vesicles and helps fuse vesicles to the membrane (Arriagada et al., 2019). Spatial trafficking of Rac1 to the leading-edge membrane controls the cell polarity that is crucial for mesenchymal migration. Rab5-mediated endocytosis is initiated by growth factors signaling, like Hepatocyte Growth Factor (HGF), at the leading-edge of a cell. HGF initiates clathrin recruitment to form a membrane vesicle. Rab5 finalizes early endosome formation and can recruit Rac1 along with Tiam1 (a Rac1-GEF). The localization of Rac1 and Tiam1 at the early endosome increases their

interaction and favors the active Rac1 conformation (Palamidessi et al., 2008). Colocalization of Rac1 and Tiam1 is increased when cells are placed in a stiff matrix (Palamidessi et al., 2008). Rac1 is activated and recycled at leading-edges through endosomes creating a signaling gradient, which is a hallmark of mesenchymal migration. Impairing Rab5 endocytosis will drastically impede Rac1 activity and display a round-amoeboid phenotype (Palamidessi et al., 2008). Although the exact scaffolding mechanism of active Rac1 and Tiam1 to leading-edge endosomes is not known, Rab5 is still a determinate in polarization rac1, and consequently RhoA, through its double-negative network. Rab5 is also reported to be dependent on active-integrin complexes at the leading-edge (Arriagada et al., 2019). Loss of adhesion complexes under soft matrix conditions may promote MAT by prevent Rac1 trafficking and polarization to the leading-edge.

Local degradation of Rho GTPases can also help establish cell polarity, shape, and behavior. Smurf1 is an enzyme that can degrade RhoA and decrease cortical contractility (Sahai et al., 2007). Smurf1 is recruited by a Cdc42-dependent polarity complex comprising of PAR6-aPKC (Sahai et al., 2007). Cdc42 is predominately localized at the leading-edge of mesenchymal cells, like Rac1. If there is a loss of cell polarity, the PAR6-aPKC complex will fail to form and will not activate Smurf1 (Sahai et al., 2007). RhoA will not be degraded and can now favor the amoeboid mode. The most common way cells can lose their polarity is if there is a loss of adhesion (cell-cell or cell-ECM), and that is most frequently seen when cells are in a soft matrix, due to the lack of stable ligands (Gandalovičová et al., 2016).

We saw the importance of integrin in Rac1 activation, so it is reasonable to speculate that integrin turnover affects the signaling of Rac1 and therefore Rho in a cell (Carragher et al., 2006). At active focal adhesions, FAK is recruited to activate Src kinase. Src kinase is able to act on calpain-2, a proteolytic protein that cleaves focal adhesion components (such as FAK, talin, actinin, integrin, and paxillin) resulting in disassembly of the adhesion complex (Westhoff et al., 2004). Integrin disassembly is necessary to create new traction point during mesenchymal

migration. Inhibition of calpain-2 will prevent new integrin adhesion and the cell will remain immobile. Lack of integrin complexes impairs Rac1 activation and its negative feedback on RhoA, allowing a higher cortical contractility and imposes an amoeboid phenotype (Carragher et al., 2006). Calpain-2 can modulate the activity of Rac1, and in low adhesion condition can help promote MAT.

Aside from adhesion signaling or chemical signaling, another possible regulatory component of the RhoA/Rac1 network is through mechanosensing membrane proteins. Syndecan-4 is a membrane proteoglycan that functions to transiently sense ECM components like collagen (Elfenbein and Simons, 2013). Syndecan-4 is not an adhesion molecule. It is a transmembrane protein that can transduce environmental cues to the cytoskeleton by intracellularly binding to Src, cortactin, and tubulin (Elfenbein and Simons, 2013). As the cell receives the signaling, it causes remodeling of the actin cytoskeleton to optimize the migration, like MAT. This is achieved through syndecan's modulation of RhoA regulation (Elfenbein and Simons, 2013; Helen K. Matthews 2008). Rac1 can inhibit syndecan signaling, but in the absence of focal adhesion signaling, Rac1 activity is reduced. Under low adhesion, syndecan can move to the membrane to further reduce activity of Rac1 and promotes RhoA activity, in favor of the amoeboid phenotype (Helen K. Matthews 2008).

MAT can be seen when tumor mesenchymal cells are placed in a soft ECM with no adhesion. This adaptation is thought to be mediated by perturbed membrane signaling (Killaars et al., 2019) or by altering adhesion signaling (Liu et al., 2015; Čermák et al., 2018). These changes in adhesion and membrane signaling causes diverse effects on various signaling programs which ultimately modulate the RhoA and Rac1 double-negative network. These pathways are consistent with our model in low adhesion. Next, we will see how high confinement, along with low adhesion, can also enhance cortical contractility to influence MAT.

Bistable Signaling in Amoeboid Cells in High Confinement and Low Adhesion Environments

Low adhesion conditions favor mesenchymal to amoeboid transition in transformed cells. The cells are switching modes of migration solely based on differences in environmental parameters. Recent research has shown that if the cells experience mechanical pressure, from being in a confined space, mesenchymal cells can transition into the amoeboid phenotype, as well (Liu et al., 2015). Confinement in a tumor environment can be developed from high cell density regions due to uncontrolled replication, and from an in increase collagen deposition and crosslinking proteins by cancer associated fibroblast (CAF) or other tumor cells (Friedl and Wolf, 2010). A possible mechanism for MAT in confinement may involve some mechanical regulation of the double-negative RhoA/Rac1 module in order to favor RhoA-ROCK-myosin activity without gene expression changes or adhesion signaling.

When a cell is in a confined space the membrane experiences an increase in tension as it is being stretched and squeezed (Hung et al., 2016). Consequently, it affects mechanically-sensitive membrane proteins. One example is the membrane bound calcium cation stretch channel, PIEZO1, which is sensitive to membrane stretching and can increase cortical myosin organization when activated (Figure 13) (Hung et al., 2016). In response to increased

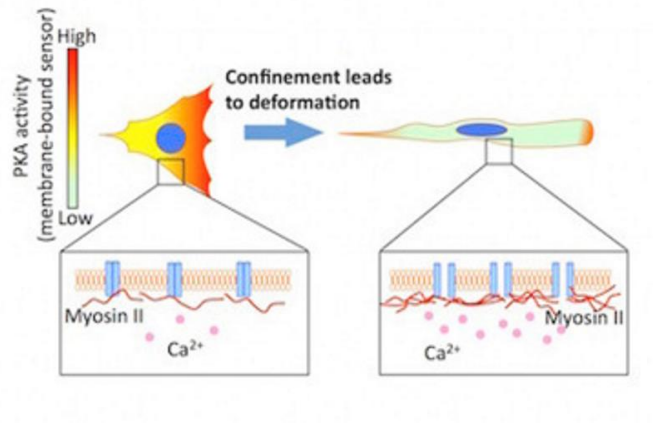

Figure 13: Confinements causes quick activation of calcium stretch channels, PIEZO1. PEIZO1–Ca2+ release deactivates PKA. PKA directly phosphorylates RhoA and or RhoGEF for inhibition. Inhibting PKA will increase RhoA and increase contractility for amoeboid migration. Hung, W.-C., Yang, J.R., Yankaskas, C.L., Wong, B.S., Wu, P.-H., Pardo-Pastor, C., Serra, S.A., Chiang, M.-J., Gu, Z., Wirtz, D., et al. (2016). Confinement Sensing and Signal Optimization via Piezo1/PKA and Myosin II Pathways. Cell Reports 15, 1430-1441. Permission by Creative Commons; https://creativecommons.org/licenses/by/4.0/

membrane tension, the PIEZO1 channels open and increase intracellular Ca2+ which activates of PDE1, a phosphodiesterase to deactivate PKA (Figure 13) (Hung et al., 2016). PKA directly phosphorylates either RhoA or Rho-GEF, causing inhibition of these proteins (Hung et al., 2016). The double-negative relationship between Rac1 and RhoA allows Rac1 to be active when RhoA is inhibited by PKA. During confinement, the increased intracellular calcium prevents PKA from inhibiting RhoA, and pushes the equilibrium towards RhoA-ROCK module for increased myosin contractility (Figure 13). Moreover, myosin can regulate other mechanosensing mechanisms which leads to enhancement of the initial contractility (Jacobelli et al., 2010). Myosin can also inhibit PKA and negatively regulate Rac1 (Hung et al., 2016). Additionally, Ca2+ influx can also directly enhance myosin contractility through calmodulin singling, which may contribute to maintenance of a cell transition state under confinement (Hung et al., 2016). Overall, PEIZO stretch channels link the increased RhoA-dependent-myosin activity with increasing cell confinement.

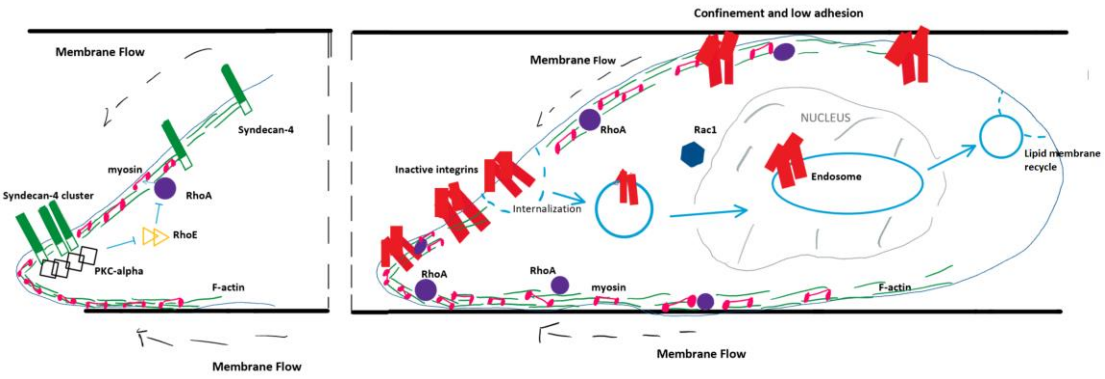

Figure 14: i) Rear section of cell in low adhesion and confinement cell. Syndecan-4 is transported to the rear of the cell creating protein clusters that faiciliate increased activation of PKCa. PKCa then inhibits RhoE which reduces inhibtion on RhoA at cortical memrbane. Overall, there is increased RhoA-dependent myosin localization on cortical memrbanes as a result of a high confinement and low adhesion environemnt.

ii) Memebrane flow of a cell in a confined, low adhesion evnironment. Membrane and transmembrane proteins like integrin, flow rearward. Inactive integrins are internalized to be recycled, preventing Rac1 activation and favoring RhoA actviation as a result of their double-negative relationships.

Another way tumor cells can mechanically regulate MAT may be through syndecan-4 regulation. Syndecan-4 can inhibit RhoE signaling through PKCa which results in prolonged activity of RhoA (Elfenbein and Simons, 2013). RhoE is a membrane-bound GTPase that directly

inactivates ROCK kinase via binding, which will limit ROCK interaction with myosin. Inhibition of RhoE leads to increase actin-myosin contractility that is seen in bleb protrusion retraction (Riento et al., 2003). A possible regulator upstream of RhoE is PKCa. PKCa is a membrane-bound protein that is controlled by syndecan-4 signaling (Nakashima, 2002). PKCa also controls the uptake of integrin which is used for focal adhesion signaling (Elfenbein and Simons, 2013). As integrin is endocytosed, syndecan signaling overshadows integrin activity, favoring RhoA activation (Elfenbein and Simons, 2013). Tumor amoeboid cells in high confinement spaces will exhibit increased retrograde flow of the membrane and transmembrane proteins to facilitate cell displacement. Syndecan-4 could be transported to the rear of the cell leading to aggregated clusters of sydencan-4; these clusters can then overtly recruit PKCa (Figure 14i). Increased localization of PKCa to the membrane is activated by diacylglycerol (DG) or Ca2+ release (Nakashima, 2002). PKCa recruits and concentrates membrane-bound RhoE for inhibition (Nakashima, 2002). Therefore, there will be an overactivation of RhoA as a result of the high confinement environment (Figure 14i).

The increased membrane flow, in confinement and low adhesion, may also translocate inactive integrin proteins to the rear, allowing integrin internalization as the cell tries to recycle the lipids from the membrane, back to the front of the cell to continue the retrograde cycle (Figure 14ii) (O'Neill et al., 2018). Endocytosis of inactive integrins could enhance in loss of Rac1 and increase of RhoA (Figure 14ii) (Byrne et al., 2016). Overall, low adhesion and high confinement causes MAT in tumor cells through RhoA and Rac1 signaling module, in support of the low adhesion model (Figure 1 and Figure 14). If we can to control the regulation of these RhoGTPases then it could be used in antitumor therapies to prevent MAT and deter tumor invasion.

Conclusion

In vivo, tumor cells are exposed to a complex tumor environment. The environments the tumor cells experiences are not so well defined like in experimental parameter: having only stiff or soft matrix, and having high or low confinement, but rather it is a mixture of all these variables. We saw that each mode of migration is generated when the tumor cells are introduced into their respective conditions, but will it remain in its newly transformed phenotype or will it continuously change if tumor cells are in this always-changing environment? A group recently proposed a gradient model, where at the extremes of these confinement and adhesion conditions, there is the distinct amoeboid migration with dominant RhoA at one end, and mesenchymal migration with dominant Rac1 at the other end (Figure 15) (Holle et al., 2019). This consistent with our low-adhesion model controlling RhoGTPase. In-between these extreme points, however, lies an intermediate mode exhibiting both amoeboid and mesenchymal characteristics with balanced Rac1 and RhoA activity. This idea conflicts with our model, positing that it is not a binary switch between extreme RhoA and Rac1 as explained before, but rather, a gradient. Perhaps this suggests that there is intrinsic regulation that finely tunes RhoGTPase activity, instead of pushing towards one extreme like what we have explored with low adhesion and confinement. We have yet to explore if intermediate levels of adhesion can exhibit mixed phenotype as seen by Holle et al. (Figure 15). Understanding how these

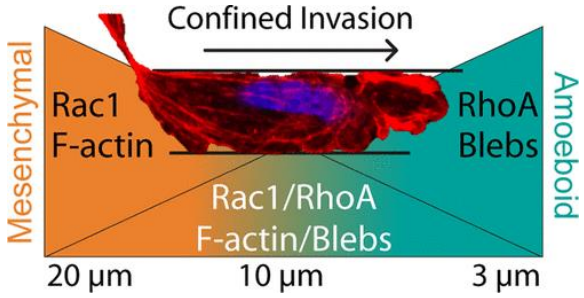

Figure 15: A gradient model, where at the extremes of these confinement and adhesion conditions, there is the distinct mesenchymal mode and at the other end is the amoeboid mode. In between, there is an intermediate mode exhibiting both amoeboid and mesenchymal characteristics. Holle, A.W., Govindan Kutty Devi, N., Clar, K., Fan, A., Saif, T., Kemkemer, R., and Spatz, J.P. (2019). Cancer Cells Invade Confined Microchannels via a Self-Directed Mesenchymal-to-Amoeboid Transition. Nano Letters 19, 2280-2290. Permission by Creative Commons; https://creativecommons.org/licenses/by/4.0/

intermediate cells (under a mixed low/high confinement condition) preferentially choose their mode of migration may be the next step in understanding how cell confinement and adhesion contribute to cell plasticity through RhoGTPase regulation.

Appendix

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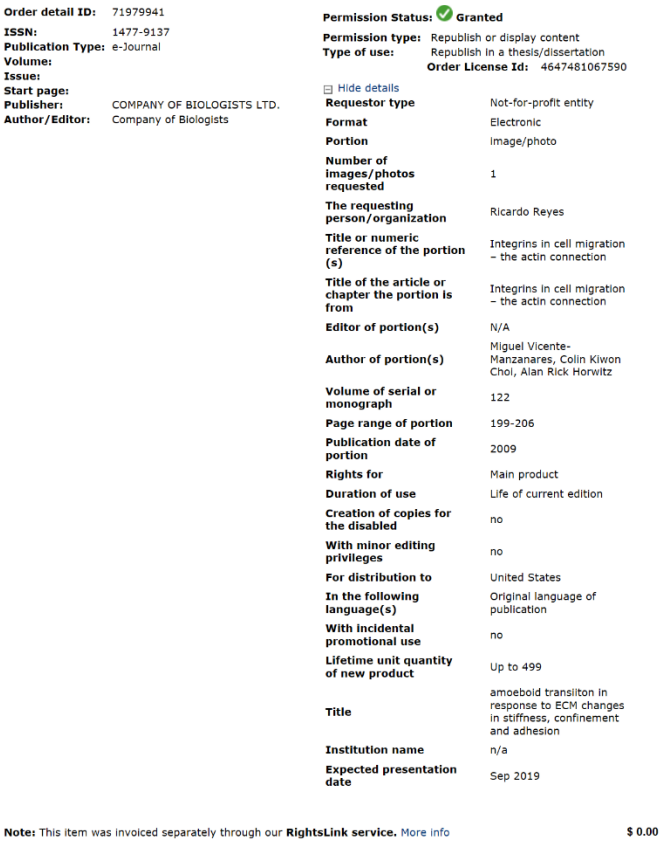

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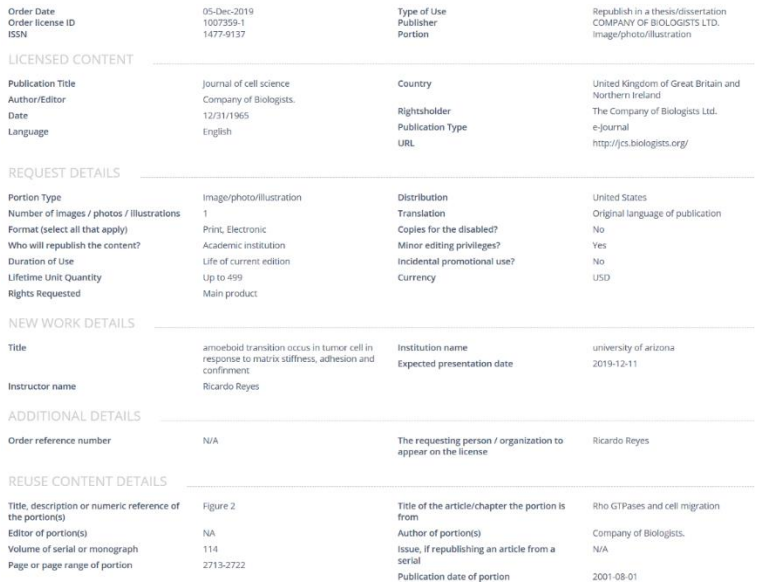

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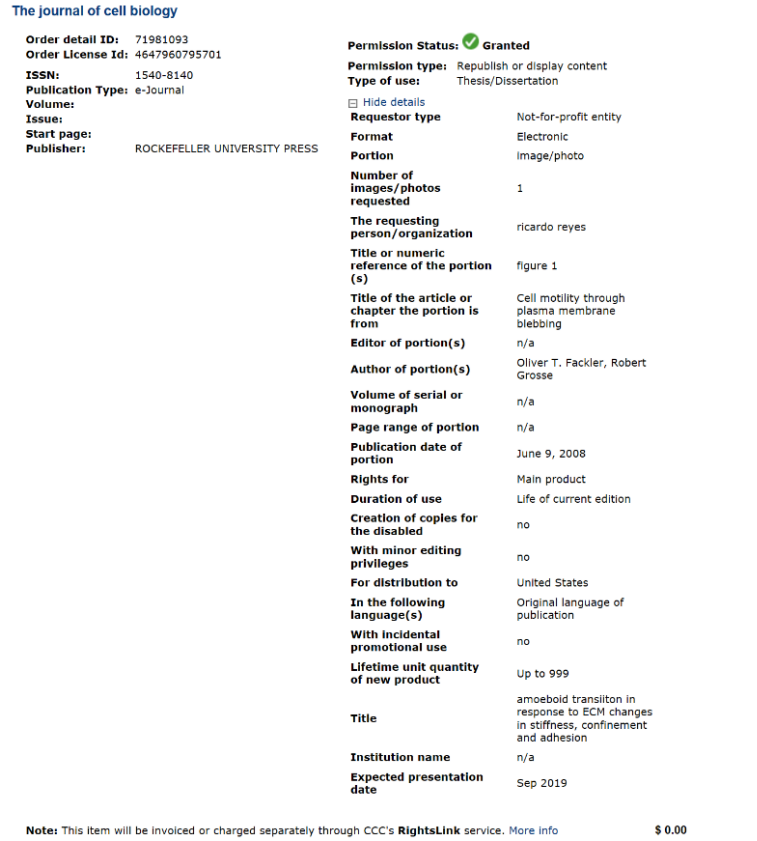

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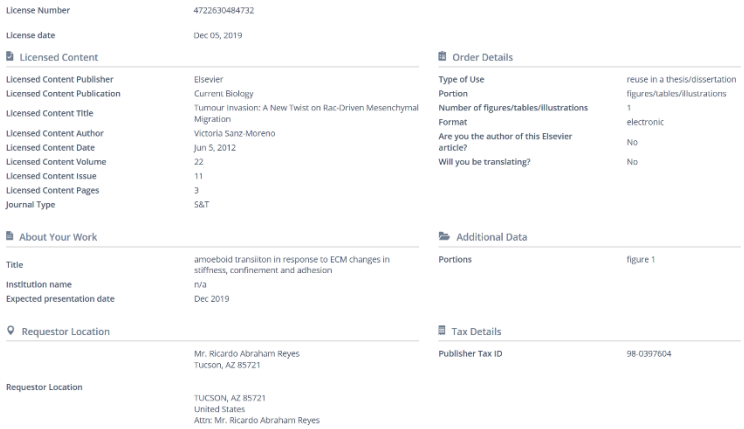

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