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The role of cell polarity signaling in the plasticity of cancer cell invasiveness

Úloha signalizace regulující buněčnou polaritu v plasticitě invazivity nádorových buněk

Bachelor's thesis

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Declaration:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 12. 5. 2014 Aneta Gandalovičová

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Contents

List of abbreviations:

ADAM	A Disintegrin and Metalloproteinase
AJ	Adherens junction
AMT	Amoeboid-to-mesenchymal transition
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
aPKCζ	Atypical protein kinase C isoform zeta
aPKCι	Atypical protein kinase C isoform iota
ARHGAP22	Rho GTPase-activating protein 22
Arp 2/3	Actin-related proteins 2/3 complex
CAS	Crk-associated substrate
CAT	Collective-to-amoeboid transition
Cdc42	Cell division control protein 42
Crk	Adapter molecule crk
Crumbs	Crumbs homolog
DIAPH3	Diaphanous-related formin 3
Dlg	Disk large homolog
DOCK3	Dedicator of cytokinesis 3
DOCK10	Dedicator of cytokinesis 10
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
Erb2	Receptor tyrosine-protein kinase erbB-2
ERM	Ezrin-radixin-moesin
F-actin	Filamentous actin
GAP	GTPase-activating protein
GDI	GDP Dissociation Inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GSK-3β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
JAM	Junctional adhesion molecule
L27	Lin-2 and Lin-7 protein domain
Lgl	Lethal giant larvae
LIMK	LIM kinase
LRR	Leucine rich repeat
MAT	Mesenchymal-to-amoeboid transition
MET	Mesenchymal-to-epithelial transition
MLC	Myosin light chain
MLC2	Myosin light chain 2
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
MRCK	Myotonic dystrophy kinase-related Cdc42-binding kinase
MTOC	Microtubule-organising centre
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9

NG2	Neural/glial antigen 2
PAK1	P21-activated kinase 1
Pals1	Protein associated with Lin-7
Par	Partioning defective
Par1	Partioning defective homolog 1
Par3	Partioning defective homolog 3
Par6	Partioning defective homolog 6
PATJ	PALS1-associated TJ protein
PDGF	Platelet – derived growth factor
PDZ	Postsynaptic density protein-95, disc-large, zonula occludens-1 domain
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol(4,5)bisphosphate
PIP3	Phosphatidylinositol(3,4,5)triphosphate
pRB	Retinoblastoma-associated protein
PTA	Podosome-type adhesion
PTEN	Phosphatase and tensin homolog
Rac1	Ras-related protein Rac1
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated, coiled-coil containing protein kinase 1
SCAR	Suppressor of cAMP receptor
Scribble	Scribble homolog
siRNA	Small interfering RNA
Smurf1	SMAD specific E3 ubiquitin protein ligase 1
Stat3	Signal transducer and activator of transcription 3
TGFβ	Transforming growth factor beta-1
Tiam1	T-lymphoma invasion and metastasis-inducing protein 1
TJ	Tight junctions
VEGF	Vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family protein member
ZEB1	Zinc finger E-box-binding homeobox 1
ZIPK	Zipper-interacting protein kinase
ZO-1	Zonula occludens protein 1
βPIX	PAK-interacting exchange factor β

Abstract and keywords:

Throughout the last few years cancer research has focused on studying the origin of secondary tumors, i.e. metastases, which are a direct outcome of the ability of cancer cells to disseminate from the primary tumor and invade the adjacent tissue. Generally, cancer cells migrate by two distinct mechanisms- amoeboid or mesenchymal. Whereas the mesenchymal migration mode can be described as "path generating", the amoeboid mode resembles a "path finding" way of migration. Both types of invasion are regulated by divergent signaling pathways that are closely related to cell polarity and cytoskeleton reorganization. Responsible for cell polarization are not only the polarity complexes Par, Scribble and Crumbs, but also phosphoinositides and Rho GTPases Rac, Rho and Cdc42, which, additionally, regulate the dynamics of the cytoskeleton. By a mutual interplay they regulate cell motility. It cannot come as a surprise that their deregulation commonly results in tumorigenesis. A more thorough comprehension of the signaling pathways leading to cancer cell invasiveness is a necessary step towards understanding the complex problem of metastasis.

Key words: invasiveness, amoeboid, mesenchymal, cell polarity, motility, Rho GTPases, polarity complexes

Abstrakt a klíčová slova:

Výzkum rakoviny se v posledních letech zaměřil na vznik sekundárních nádorů, tj. metastáz, které jsou přímým důsledkem schopnosti nádorových buněk opustit primární nádor a invadovat do okolní tkáně. Rakovinové buňky migrují především dvěma rozdílnými mechanismy- améboidním a mezenchymálním. Zatímco mezenchymální způsob migrace lze popsat jako „cestu vytvářející", améboidní připomíná spíš „cestu hledající" migraci. Oba typy invazivity jsou regulovány odlišnými signálními dráhami, které úzce souvisí s buněčnou polaritou a přestavbou cytoskeletu. Za polaritu buňky jsou zodpovědné nejen polarizační komplexy Par, Scribble a Crumbs, ale také fosfoinositidy a Rho GTPázy Rac, Rho a Cdc42, které navíc řídí dynamiku cytoskeletu. Vzájemnou souhrou regulují buněčnou motilitu. Není proto překvapením, že jejich deregulace často vede ke karcinogenezi. Dokonalejší prozkoumání signálních drah vedoucích k buněčné invasivitě je nutným krokem k porozumění komplexního problému vzniku metastáz.

Klíčová slova: invazivita, améboidní, mezenchymální, buněčná polarita, motilita, Rho GTPázy, polarizační komplexy

1. Introduction

Cell migration is an essential process in every multi-cellular organism. It is required not only during development but also wound healing or the immune responses. These processes are carefully controlled within the organism because excess of cell migration can cause the disintegration of tissues, which is exactly what happens during cancer. When cells generate enough mutations to overcome surveillance they can start to uncontrollably proliferate, thus becoming cancer cells. Some of these cells loose contact inhibition and migrate to distinct places where they can form a new tumor. This negative phenomenon is called metastasizing and the ability of cells to move through the extracellular matrix is referred to as invasiveness. For directional movement cells need to be able to recognize the front from rear, which is possible due to a gradient of certain molecules and/or the specific localization of cell polarity proteins. Unsurprisingly, the molecular mechanisms of cell migration and cell polarity are densely interconnected. They both function through cytoskeleton remodeling, both depend on certain adhesion molecules and both have a direct link to cancer. Recently, many reviews about the influence of cell polarity on cancer have been published while other have covered the topic of cell migration. However, not much published work describes the influence of cell polarity on individual types of cancer cell invasiveness.

At the present, finding a cure for cancer is the Holy Grail of science and medicine. Many therapeutic agents have been established, most often blocking proliferation of the tumor. Unfortunately, even today we are not able to efficiently block cell invasion and metastasis. The necessity for deeper understanding the molecular mechanisms of invasiveness is evident.

In this thesis I shall review and summarize up to date information on the role of cell polarity in cancer cell invasiveness. The main goals are as follow:

1. **Review cell polarity proteins and the mechanisms of polarization.**
2. **Describe cancer cell invasion, emphasizing the different molecular mechanisms of each invasion mode.**
3. **Summarize current knowledge of the interplay between cell polarity and cell motility.**
4. **Consider the role of proteins regulating polarity in cancer cell metastasis and reflect their possible therapeutic impact.**

2. Cells that kill

The one deadly disease affecting people all over the world of all ages, one which scientists have been trying to defeat for many decades, is cancer. It is striking that cancer is in fact a result of malformed cells within our own body. These cells represent a mortal danger when left uncontrolled.

During their lifespan, cells can accumulate mutations and genomic instability in such an extent, that it leads to cell death or, under certain circumstances, cell transformation. Overtime, the transformed cell can progress into a primary tumor, which is either classified as benign or malignant. If cells disseminate from a tumor mass and invade the surrounding tissue, the tumor is usually distinguished as malignant, mainly meaning it is capable of metastasis. Contrary, a benign tumor is in most cases incapable of invasion, although many benign tumors become malignant over time. Malignant tumors are justly feared given it is the secondary tumors responsible for death in the majority of cases (Sporn, 1996).

The process during which a cell transforms into a cancer cell has been reviewed by Douglas Hanahan and Robert Weinberg, who postulated the today generally accepted hallmarks of cancer (Hanahan & Weinberg, 2011):

1. sustaining proliferative signaling
2. evading growth suppressors
3. ***activating invasion and metastasis***
4. enabling replicative immortality
5. inducing angiogenesis
6. resisting cell death
7. deregulating cellular energetics
8. avoiding immune destruction
9. genome instability and mutation
10. tumor- promoting inflammation

This thesis is restricted to discussing the third hallmark: activating invasion and metastasis. It is essential to realize that establishing front-rear polarity precedes invasion, so to fully understand cell invasiveness, one has to first comprehend the mechanisms of cell polarization.

3. Cell polarization

It is common knowledge that polarization and correct placement of each cell is essential for tissue integrity and homeostasis, especially in epithelial tissues. The cytoskeleton distribution, cell polarity complexes, cell-cell adhesions and contact with the ECM (extracellular matrix) all contribute to the fact that cells remain polarized. Migrating cells exhibit an apparent front-rear polarity and the apico-basal polarity is most evident in epithelial cells. It is determined by specific localization of cell adhesions- tight junctions (TJ) and adherens junctions (AJ). Tight junctions, which can be found at the apical side, consist of proteins, such as occludin, claudin and JAMs (junctional adhesion molecules) plus associated cytoplasmic scaffolding proteins, for example ZO-1 (short for zonula occludens 1). On the other hand, AJs mediate cell-cell contact and are mostly composed of E-cadherin and catenins (p120-catenin, β-catenin and α-catenin). The band of AJ is called zonula adherens and divides the cell into apical and basal regions.

Conditions leading to the loss of cell polarization or aberrant cell polarization can induce an invasive phenotype and result in malignant progression. Interestingly, the three protein complexes (Par, Scribble and Crumbs) responsible for establishing apico-basal polarity are very well preserved among metazoa proving their importance. They are known to play a key role in developmental processes, but also, as recent studies have confirmed, they contribute to the regulation of malignant progression (reviewed in Ellenbroek et al., 2012). Generally they are considered to be tumor suppressors (Karp et al., 2008; reviewed in Bilder, 2004). Signaling pathways of the Par, Scribble and Crumbs are not only mutually interconnected, but also associated with small GTPases- GTP (guanosine triphosphate) binding proteins important for cytoskeleton regulation and thus front rear polarization.

3.1. Cell polarity complexes

The range of polarity proteins is the underlying element of variable cell morphologies. Moreover, they can either support one another, strengthening the signal, or antagonize each other, eliminating a certain stimulation. Their spatial and temporal distribution orchestrates cell polarity. This chapter is dedicated to polarity protein complexes, whilst chapter 3.2. describes Rho GTPases and their role in cell migration and invasion. Chapter 3.3. briefly introduces the role of phosphoinositides in polarity.

3.1.1. The Par complex

Out of the three polarity complexes, Par has the widest range of functions. Par is short for Partioning defective, a protein complex first identified during studies of *Caenorhabditis elegans* development. The complex is located at the apical side within the tight junctions region and consists of Par3, Par6

and aPKC, which stands for atypical protein kinase C. While Par3 and Par6 play the role of PDZ[1] binding domain scaffold proteins, aPKC (either aPKCι or aPKCζ) is an important polarity regulator. As a kinase, it phosphorylates proteins, a modification which often leads to change of substrate protein localization and/or activity. Moreover, it can directly bind to both Par6 and Par3 (Izumi et al., 1998; Joberty et al., 2000). The whole complex Par3-aPKC-Par6 is needed to form the apical membrane (Horikoshi et al., 2009). Unlike the apically localized duo Par6-aPKC, Par3 is targeted primarily to the tight junctions by associating with JAM (junctional adhesion molecule) (Itoh et al., 2001).

3.1.2. The Crumbs complex

Along with Par, the Crumbs complex also localizes to the apical side, particularly to the apical membrane. It consists of proteins Crumbs, Pals1 (protein associated with Lin seven 1) and PATJ (Pals1 associated tight junction protein). Through its PDZ protein binding domain Crumbs binds to Pals1 and contributes to tight junction formation (Bachmann et al., 2001). Furthermore, Pals1 itself binds PATJ through a L27[2] domain (Li et al., 2004). Finally, PATJ is a scaffold protein with many interactions via its PDZ domain that has been shown to take part in regulating tight junctions formation (Straight et al., 2004) but also cell migration (Ernkvist et al., 2009)

3.1.3. The Scribble complex

Unlike the previous two complexes, Scribble is localized basolaterally. By being mutually antagonist to Crumbs and Par, the apico-basolateral polarization is established. The Scribble complex proteins are Scribble, Dlg (short for Disc large) and Lgl (short for Lethal giant larvae). The unusual names originate from the mutated proteins' effect during *Drosophila melanogaster* development.

Like Par3, Par6 or Crumbs and PATJ, also both Scribble and Dlg contain PDZ domains. Scribble also contains a LRR (leucin rich repeat) sequence, which intermediates direct interaction between Scribble and Lgl (Kallay et al., 2006). The binding of Scribble to Dlg is indirect (Mathew et al., 2002). Further, by its PDZ domain Scribble binds vimentin. What is more, through its PDZ domains Dlg can bind APC (Adenomatous polyposis coli), PTEN (Phosphatase and tensin homolog) and the proto-oncogene β-catenin (Etienne-Manneville et al., 2005; Sotelo et al., 2012; Subbaiah et al., 2012).

3.1.4. Mutual interactions

Throughout the years of studying cell polarity complexes many interactions among the proteins involved in establishing cell polarity have been revealed. Crucial is the regulation of basolateral

[1] The PDZ binding domain is a conserved binding sequence found in many proteins, all known protein-ligand interactions are listed in the online available database PDZbase (http://abc.med.cornell.edu/pdzbase/). PDZ stands for postsynaptic density-95, discs large and zonula occludens 1 (ZO-1).
[2] L27 is another protein binding domain. It can be found in the proteins Lin2 and Lin7, which gave the domain its name. It serves mainly as a interaction mediating module.

localization of the Scribble complex by aPKC- when Lgl translocates to the apical side, it is phosphorylated by aPKC, a modification resulting in re-location from the apical region (Plant et al., 2003). On the other hand, the apical complexes are expelled from the basal region by Par1, a kinase localized laterally. Par1 phosphorylates Par3/Par6 leading to inhibited formation of the functional Par complex with aPKC (Benton and Johnston, 2003).

Furthermore, a direct interaction between Par6 and Pals1 links the Crumbs complex to the Par complex (Hurd et al., 2003). The siRNA silencing of Pals1 correspondingly led to the decrease in its binding partner PATJ, which resulted in a deregulated interaction between the Crumbs complex and the Par complex. In effect, this disrupted tight junctions and delocalized aPKC. These findings propose (PALS1-PATJ)-Par6 interaction to regulate aPKC location to the tight junctions region (Straight et al., 2004). Moreover, aPKC seems to be the key mediator in establishing apico-basal polarity. It not only keeps Lgl at the basal side, but also maintains the localization of Crumbs complex at the apical region by phosphorylating it (Sotillos and Díaz-Meco, 2004).

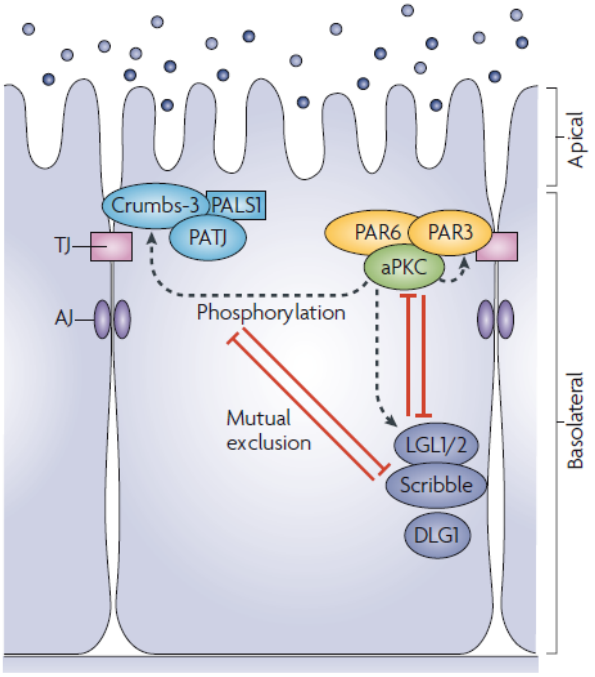


Figure I: The main interactions of the cell polarity complexes. For description see text. Image was adapted from (Iden and Collard, 2008)

Overall, the resulting polarity is an outcome of mutual interactions between individual proteins of the Par, Crumbs and Scribble complex. To be localized correctly they require one another. The main interactions are summarized in figure I.

3.2. GTPases at the crossroads of cell polarity signaling

Cell migration and polarity both include signaling through RhoA, Rac and Cdc42- all belonging to the family of small Rho GTPases, part of the Ras superfamily. They are post-tranlastionally modified at their C- end by an isoprenyl group, enabling membrane targeting. When it comes to cell motility, Rho GTPases are the critical effectors as they are essential for cytoskeleton remodeling, adhesion, and contractility (Nobes and Hall, 1999). Generally, Rho GTPases are able to transmit information about the stiffness of the ECM (Paszek et al., 2005), which affects the choice of the invasion mode.

Unsurprisingly, they are often up-regulated in cancer and have a large impact on cancer cell invasiveness.

GTPases are often called molecular switches for their on and off state that depends on the binding of GTP, respectively GDP. Three main groups of proteins take part in the regulation of Rho GTPases:

Figure II: Proteins regulating the GTP/GDP cycle

Short	Full name	Function	Effect
GEF	Guanine exchange factor	Promotes the exchange of GDP for GTP	**activates**
GAP	GTPase- activating protein	Enhances GTP hydrolyzation	**inactivates**
GDI	Guanine nucleotide dissociation factor	Binds GDP-protein and inhibits GEF	**inhibits**

The GTP/GDP cycle regulates many signaling pathways by binding to effector proteins. In the event of binding GTP, the GTPase is activated and can transmit the signal further on. Hydrolyzing GTP to GDP results in an attenuated ability to activate effector proteins. The cycle is shown in figure III.

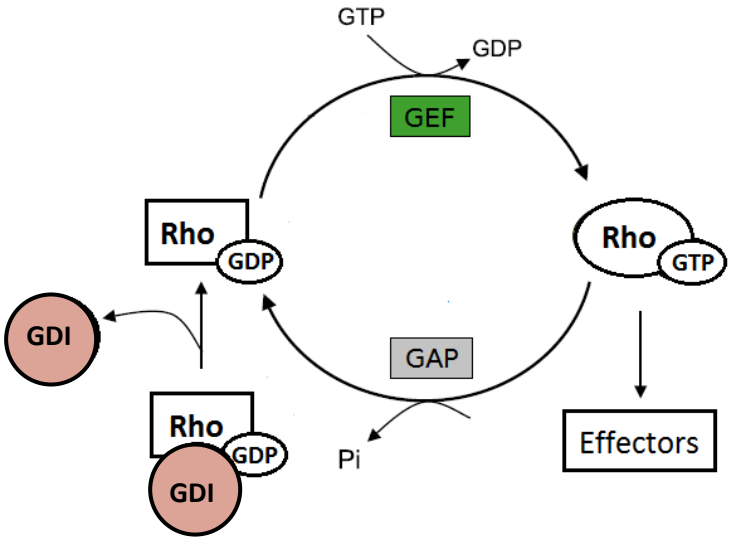


Figure III: GTP protein cycle regulated by GEF, GAP and GDI (adapted from Raftopoulou & Hall, 2004, edited). Colors correspond to figure II for easier orientation.

I have so far mentioned Rac1, Cdc42 and RhoA. However, in mammals twenty-two genes encoding Rho GTPases have been described. Among them, Cdc42, Rho isoforms A, B, C or Rac isoforms 1, 2 and 3 but also the anatagonist of RhoA/B/C RhoE. The three GTPases Rac1, Cdc42 and RhoA are most commonly studied; therefore they will be discussed in more detail unlike the rest. Briefly, the GTPases Rac1 and Cdc42 cooperate together at the leading edge, unlike RhoA which differs in function and localization.

3.2.1. Rac1

Rac1 localizes to the leading edge of the cell (Kraynov et al., 2000) and along with Cdc42 is the main regulator of the actin network, which is required for the formation of filopodia and lamellipodia[3]. Rac1 controls actin polymerization by regulating the Arp2/3 complex, which can induce nucleation of actin filaments. Both Rac1 and Cdc42 therefore promote cell migration by forming actin rich protrusions, although Rac1 alone is not sufficient for directional movement (Pankov et al., 2005).

3.2.2. Cdc42

Apart from its role in actin polymerization, Cdc42 plays a key role in establishing cell polarity and in effect directional migration (Nobes and Hall, 1999). It plays a prime role in chemotactic signaling and the reorientation of Golgi system and mictotubule orientation center. Among the effectors of Cdc42 we can find p21-activated kinase (PAK) (Morreale et al., 2000), Wiskott–Aldrich syndrome protein (WASP) (Rohatgi et al., 1999), Par6 (Joberty et al., 2000) or myotonic dystrophy kinase (MRCK) (Leung et al., 1998). Remarkably, Cdc42 is conserved in all eukaryotes, for example the yeast homolog is named Cdc42p.

3.2.3. RhoA

RhoA contributes to cell migration by regulating contractile forces generated by the acto-myosin network. It has been demonstrated to function counteractively of Rac. Its main effector protein is Rho-associated serine–threonine protein kinase, shortly ROCK, which can inactivate myosin light chain phosphatase (MLCP) (Kureishi, 1997; Totsukawa et al., 2004). MLCP is an inhibitor of myosin contraction, therefore inactivating it enables cell contraction. Thus, RhoA through ROCK and MLCP regulates contractile forces of the cell.

3.3. Phosphoinositides in polarity: PTEN versus PI3K

Although they are not usually classified as polarity proteins, PTEN and PI3K are key players in the signaling network regulating cell polarity and for this reason I have added them among cell polarity complexes and Rho GTPases. They are well studied for their role in directional migration.

Overall, inhibition of PTEN, which is considered to be a tumor suppressor, or overexpression of PI3K often results in cancer. They are mutually exclusive and have antagonist roles- PI3K produces the

[3] Filopodia and lamellipodia are types of membrane protrusions described in 2D environment. Both are rich of F-actin and form preferentially at the leading edge. Lamellipodia are flat and broad, whereas filopodia are thinner and more stretched forward.

second messenger PIP3 and localizes to cell membrane at the leading edge (Sasaki et al., 2004), while PTEN dephosphorylates PIP3 and produces product PIP2 (Vazquez et al., 2006).

Phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) are small lipid products that take part in signaling pathways regulating cell adhesion, membrane trafficking and motility. In migrating cells they are spatially distributed and are important inducers of front-rear polarity (Funamoto et al., 2002). The unequal distribution is a result of localized action of PI3K and, on the other hand, PTEN.

4. Cell invasion

In a physiological state, most cells are packed tightly together to form tissues, communicating between one another via cell-cell contact and surface receptors. By extensive signaling transmitting information from neighbor cells and the ECM, cells maintain their integrity (Meredith et al., 1993; reviewed in Park et al., 2000). If a cell, respectively a group of cells, begins to avoid these signals and starts specific signaling cascades leading to loss of cell adhesion it can gain the ability to invade the extracellular matrix.

For directional invasion the cells necessarily need to be polarized and capable of movement. A cell without polarization will intend to move in all directions resulting in no net movement (Pankov et al., 2005). Underlying cell polarization are the cell polarity complexes, responsible for apico-basal polarity, phosphoinositides and small monomeric G-proteins Rho GTPases Rho, Rac and Cdc42.

According to observations of motile cells, cell migration has been classified as a 5 step cyclic process (Friedl, 2004; Lauffenburger and Horwitz, 1996). This model is universal, it can be applied to all types of cell invasion with a few modifications and exceptions.

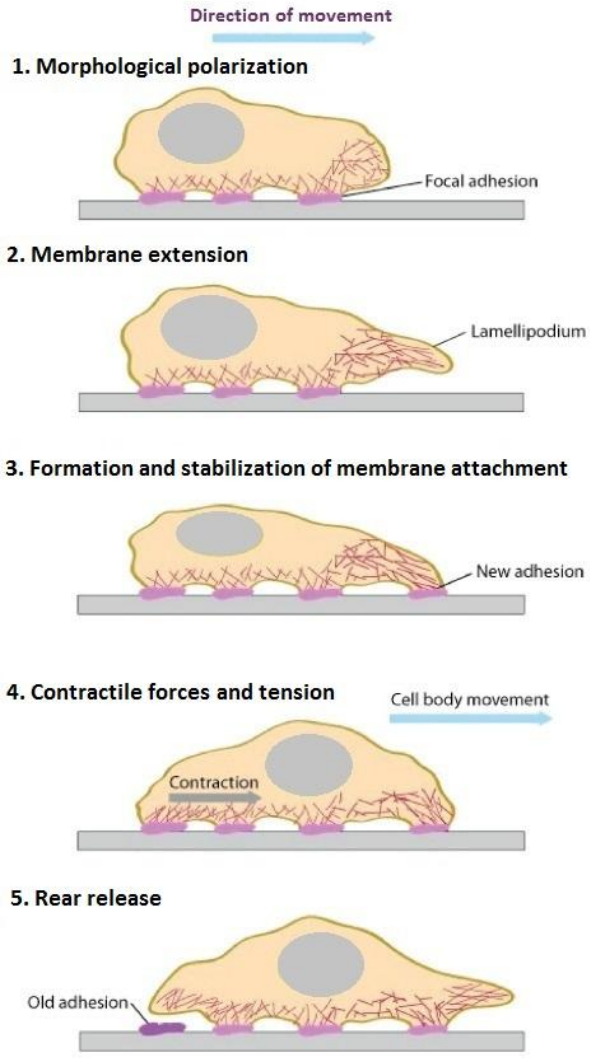


Figure IV: The 5 steps of cell movement. Image was adapted from (Ladoux and Nicolas, 2012) and modified.

Step 1 *Morphological polarization:* Cells acquire a front-rear polarization leading to a distinct morphology. This is enabled by localized filamentous actin (F-actin) distribution and results in cell asymmetry.

Step 2 *Membrane extension:* Next, the cell forms protrusions (lamellipodia, filopodia) toward the ECM at the front (leading) edge and extends matrix degrading invadosomes.

Step 3 *Formation and stabilization of membrane attachment:* Subsequently, when the protrusion gets into contact with ECM ligands, adhesion receptors (β-integrins) accumulate to form an adhesion site. The adhesion complexes are stabilized overtime, becoming focal adhesions.

Step 4 *Contractile forces and tension:* Next, the cell generates contractile forces to move directionally toward the leading edge. The contractile forces are a result of myosin II based motors moving along actin filaments.

Step 5 *Rear release:* Finally, to enable forward movement, the rear of the cells (sometimes called the uropod) must be released from the substratum. The process is referred to as "ripping" since the membrane is stripped from the adhesion complex, leaving most of the integrins behind.

Studying cancer cell invasion *in vitro* is limiting because it cannot fully imitate the conditions *in vivo*, on the other hand *in vivo* studies are less appropriate for studies on a single cell level. Modern techniques such as live cell imaging, time lapse videos or fluorescent tags enabled better tracking and imaging of individual cells. Also, creating matrices that mimic the extracellular matrix permitted to measure cell movement in 3D environment and lead to the better description of cell structures, such as invadopodia or focal complexes, as they would appear *in vivo.*

4.1. Invasion modes used by cancer cells

According to the number of invading cells, we distinguish between collective and individual invasiveness. Both have been demonstrated *in vitro* and *in vivo*. Figure V shows collective and individual invasion in an illustrative overview.

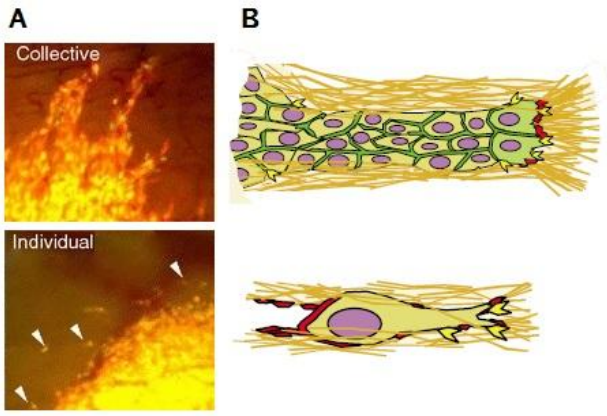


Figure V: Modes of cell invasion (upper-collective, down- individual)
A: In vivo fluorescent imaging (adapted from Alexander et al., 2008)
B: Schematic illustration (adapted from Friedl, 2004)

4.1.1. Collective cell invasion

The collective invasion mode was originally observed after histological staining when scientists recognized a cluster of cells traveling through the tissue. Collective cell migration is indispensable during morphogenesis and organogenesis, but has been also observed during metastasis. Unlike individual invasiveness, cells need to maintain cell-cell junctions (Defranco et al., 2008; Nabeshima et al., 1999), which are mainly composed of AJ, integrins and gap junctions. The cohort is polarized and

has a distinguishable front and rear (Friedl et al., 1995). The cells in the front, at the leading edge of the invading group, are called "leader", "guiding" or "pioneer" cells. They have a more dynamic actin cytoskeleton, which results in a rather mesenchymal morphology with actin rich protrusions, such as filopodia and lamellipodia. Also, leader cells have higher concentrations of surface β1-integrins compared to the cells at the rear (Hegerfeldt et al., 2002). Furthermore, these cells display higher levels of surface enzymes capable of degrading the ECM (Nabeshima et al., 2000). On the other hand, "following" cells maintain a more compact organization. The role of Rho GTPases differs in "leader" and "following" cells (Gaggioli et al., 2007). The cohort keeps together by cell adhesion molecules, especially N- and E-cadherins. Recent work demonstrates, that the cell-cell contact is strong enough to keep the migrating mass together even in heterogeneous ECM (Vedula et al., 2014). Additionally, the migrating cohort forms cell contacts with surrounding cells, called "accessory" cells. In collective cancer cell invasion, fibroblasts function as accessory cells by remodeling the ECM.

4.1.2. Individual cell invasion- the mesenchymal mode

Mesenchymal invasiveness was the first to be described and best fits the 5 step model described earlier. Typical features are cell adhesion and ECM degradation, both are indispensable for this mode of invasion. Additionally, it is characterized by an elongated morphology similar to the one of fibroblasts, often being called "spindle-like" for its long membrane protrusions.

Cells using the mesenchymal invasion mode form special adhesion structures called invadopodia and podosomes. Some studies preferably use the collective term invadosomes or podosome-type adhesion (PTA). Both podosomes and invadopodia are formed at the site of cell-ECM contact and are build of a core rich in F- actin. Other regulatory proteins such as Arp 2/3, cortactin or WASP co-localize to the center. Around the core, there is a ring of adapter proteins (vinculin and paxilin). Additionally, they contain numerous adhesion molecules. Podosomes were first described in cells of the hematopoietic lineage, such as dendritic cells or macrophages, but have been also observed in smooth muscles cells or some endothelial cells. On the other hand, invadopodia are typical for migrating cancer cells. Compared to invadopodia, podosomes are less stable, smaller and have a lower degrading capacity (reviewed in Linder et al., 2011).

The proteolytic activity is provided by enzymes capable of degrading components of the ECM- MMPs (matrix metaloproteases), ADAMs (a disintegrin and metalloproteinase), serine proteinases, and cathepsin proteinases. Most important for cancer cell invasiveness are matrix metaloproteases. The prefix metalo- refers to two zinc ions, which necessarily need to be bound to the catalytic domain for correct function. Additionally, to become activated, MMPs must be cleaved, since they are expressed as inactive pro-enzymes. Subsequently, these enzymes are recruited to the integrin- ECM

binding site of invadopodia (Artym et al., 2006) and digest the adjacent ECM making space for the cell's forward movement (Mueller et al., 1999; Wolf et al., 2003), as shown in figure VI. The ECM degradation can be observed as a tube-like matrix defect that trails the invasion pathway (Friedl and Wolf, 2008; Wolf et al., 2003). It cannot come as a surprise that up-regulation of specific MMPs in tumors has been confirmed, since many of them facilitate invasion (Lubbe et al., 2006; Poola et al., 2005).

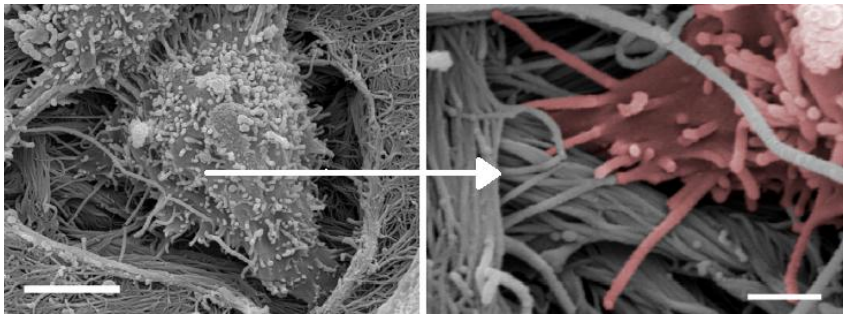


Figure VI: Left- Cell migrating in mesenchymal invasion mode visualized by scanning electron microscopy. A degraded cavity in the ECM can be seen around the cell, scale bar 5 µm. Right- Close up of invadopodia, scale bar 1 µm. Adapted from (Tolde et al., 2010)

As already mentioned, mesenchymal motility depends on the formation of adhesion complexes, mainly composed of integrins, and on the nature of the ECM. Unstable and temporary adhesions are called focal complexes, which either disassemble or maturate into stable focal adhesions (Zaidel-Bar et al., 2004). A high turnover of focal complexes correlates positively with increased cell motility and velocity. By forming adhesions at the front and releasing those at the end, the cells "crawl" forward.

Whether the cell movement is based on pushing the front or tugging on the end is the subject of current research. Both focal adhesion turnover and degradation of the ECM are limiting factors regarding the invasion speed which is approximately 0,1 - 1 µm/min (Zijl et al., 2011), which corresponds to a distance up to 4,32 cm per month.

The whole process of mesenchymal invasion relies on spatial distribution of Rho GTPases, adhesion molecules and second messengers. First, polarization of the cells causes the nucleus and microtubule organizing center (MTOC) to relocate to the rear of the cell in such a way that the MTOC is in front of the nucleus according to the direction of migration (Maninová et al., 2013). Next, the protrusions are initiated by Rac1 and Cdc42 and attach to the ECM by focal complexes. The signaling leading to protrusion extension operates through the WASP/SCAR/WAVE family of scaffold proteins which activate Arp2/3, the essential actin nucleator. While Cdc42 can bind WASP directly (Rohatgi et al., 1999), Rac activates WAVE through adaptor proteins (Eden et al., 2002). Further, surface bound MMPs degrade the ECM making space to move forward. In the meantime, the focal complexes mature into focal adhesions, which are stabilized by Rho but disassembled by Rac1.

Rac1 signaling is controlled by a positive feedback loop. The integrins from Rac-induced focal complexes activate PI3K, which localizes to the leading edge and produces the second messenger

PIP3 (Shaw et al., 1997). This second messenger is known to bind a Rac1 specific GEF, Tiam1 (short for T lymphoma invasion and metastasis), which activates Rac by GTP loading (Fleming et al., 2000). Moreover, Tiam1 is able to directly bind a subunit of Arp2/3, localizing to the actin branching point, recruiting and activating Rac1, which can subsequently activate Arp2/3, closing another positive feedback loop (Ten Klooster et al., 2006). However, there are more signaling pathways leading to Rac activation such as its interaction with DOCK3, a Rac1 specific GEF, which can interact with CAS/Crk. CAS is family of adaptor proteins in focal adhesions that form a complex with Crk, which can bind DOCK3, thus recruiting and activating Rac at the site of focal adhesions. (Sanz-Moreno et al., 2008).

Contradictory, the rear has higher concentrations of PTEN and its product PIP2 (Li et al., 2005; Vazquez et al., 2006). The main GTPase at the rear is Rho and its effector kinase ROCK, mediating contraction. ROCK phosphorylates the myosin light chain phospatase (MLCP) causing its inhibition (Kimura et al., 1996), which leads to higher myosin phosphorylation and enables generation of contractile forces needed to retract the rear end of the cell.

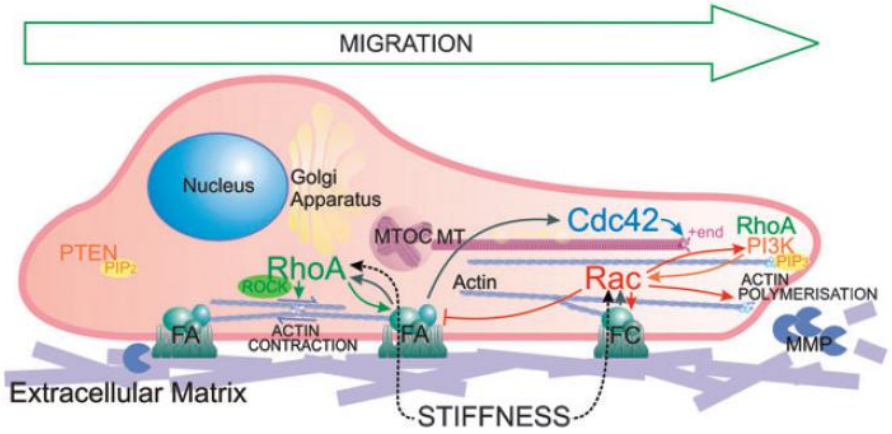


Figure VII: The distribution of polarity proteins inside a cell migrating in a mesenchymal invasion mode. Adapted from (Binamé et al., 2010)

The distribution of polarity proteins can be seen in figure VII. Thus, Rho stabilizes and induces contraction to keep the cell rear in contact with its front, while Rac initiates protrusions that tend to stretch forward. The role of Cdc42 is mainly to maintain cell polarity, i.e. directional migration.

Expectedly, the necessity of proteolytic activity for mesenchymal migration was investigated in the context of anti-cancer drugs. Inhibitors of MMPs were developed, tested and proven to block mesenchymal migration (reviwed in Hadler-Olsen et al., 2013). However, they did not turn out to be clinically as effective as it was hoped, partially because they were administered in advanced stages after metastasis had already occurred, but also due to problematic bioavailability and side effects. Nevertheless, blocking MMPs in cell lines in vitro led to the identification of the cells ability to utilize

another invasion mode- amoeboid migration (Wolf et al., 2003), its name purposely referring to the unicellular organisms Amoeba, since both easily change their shape.

4.1.3. Individual cell invasion- the amoeboid mode

Amoeboid migration is not dependent on cell adhesion. Accordingly to this, amoeboid cells have low expression of β1-integrins. The forces that result in movement are generated by contractions of cortical acto-myosin [4] (reviewed in Lämmermann & Sixt, 2009). The dynamic contractions are associated with membrane blebs, also observed in apoptotic cells. Blebs arise when the cell cortex, i.e. the acto-myosin network plus associate proteins, separates from the cell membrane by hydrostatic pressure of the cytoplasm (Charras and Paluch, 2008). Following, the pressure of the cytoplasmatic fluid expands the bleb. In non-motile cells the blebs are subsequently retracted by formation of a new acto-myosin cortex, although the precise mechanism has not yet been defined. On the other hand, migrating cells utilize the bleb to move in its direction either by forming weak, transient adhesions or by contracting the rear and pushing forward (Charras and Paluch, 2008).

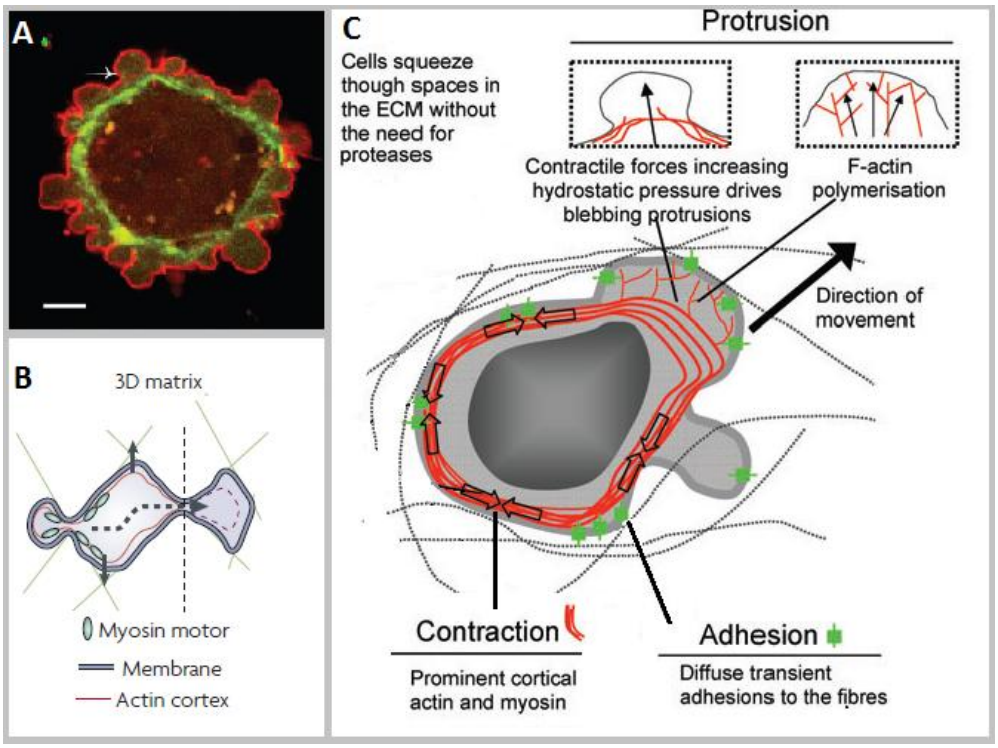


Figure VIII: **A**: Confocal microscopy image- Green : Myosin regulatory light chain localized to the cell cortex. Red: Cell membrane. Scale bar = 5 μm (Charras, 2008). **B:** Schematic picture of cells using blebs to squeeze through a 3D matrix (adapted from G. Charras & Paluch, 2008) **C:** Image illustrating the generation of forces needed for movement (adapted from Pinner & Sahai, 2008. Image was edited.)

[4] Cortical acto-myosin or simply the cell cortex, is a network of branched actin filaments and myosin motors under the cell plasma membrane, attached to it by anchor proteins.

Notably, amoeboid invasiveness is thought to be independent of matrix degradation (Fagan-Solis et al., 2013; Rösel et al., 2008; Wolf et al., 2003). Instead the cells are adaptable in shape enough to squeeze through the surrounding filaments, schematically illustrated in figure VIII. The fast deformability leads to one magnitude higher invasion velocities compared to mesenchymal invasion (Sahai and Marshall, 2003). In fact, cancer cells disseminating from a primary tumor have been shown to migrate at the speed of 15 μm/min (Pinner and Sahai, 2008)

The most prominent signaling pathway in amoeboid migration is Rho/ROCK. Upon activation by Rho, ROCK can regulate contractile forces by modulating the light chains of myosin. One of its downstream targets is the myosin light chain kinase, MLCK in short. ROCK phosphorylates MLCK (Amano et al., 1996), which subsequently phosphorylates the myosin II light chain (MLC2) and activates it. On the other hand, the antagonist myosin light chain phosphatase, shortly MLCP, dephosphorylates MLC2. Its function is inhibited after phosphorylation by ROCK (Kimura et al., 1996), also by zipper-interacting protein kinase (ZIPK) (Hagerty et al., 2007), a downstream effector of ROCK, or by myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), which is activated by Cdc42 (Wilkinson et al., 2005). Thus, both phosphorylation of MLCK or MLCP leads to the phosporylated MLC2. In this state, MLC2 interacts with actin and activates the myosin ATPase leading to contraction mediated by the myosin II motor complex.

Conversely, Cdc42 and Rac1 trigger p21-activated protein kinase 1 (PAK1), which directly inhibits MLCK via phosphorylation, resulting in decreased contractility (Sanders et al., 1999). Cdc42 also activates PAK2, although this kinase acts opposite of PAK1 and promotes amoeboid motility through activation of acto-myosin contraction (Gadea et al., 2008).

Notably, ROCK also contributes to the localization of MLC into bundles at the leading edge cell cortex, perpendicular to the direction of movement, which is necessary for force generation (Wyckoff et al., 2006). All the interactions are summarized in figure IX.

Altogether, RhoA activation of ROCK is crucial for cell contractility. ROCK enhances MLC2 phosporylation by activating MLCK and inhibiting MLCP. The overall balance between phosphorylted MLC2 and un-phosphorylated MLC2 determines the final contracting force.

Also, the spatial distribution of PTEN, PI3K and their products is essential for amoeboid motility. PI3K in amoeboid migrating cells localizes to the cell front edge without any need of a chemoatractant (Funamoto et al., 2002). At the rear, PTEN co-localizes with myosin II (Pramanik et al., 2009). Although PTEN has not been shown to regulate myosin light chain II directly, its product PIP2 is known to regulate the localization of ERM (ezrin-radixin-moesin) proteins which influence membrane-cortex adhesions. Furthermore, ERM proteins can bind to RhoGDI, which results in attenuated inhibition of Rho (Takahashi et al., 1997).

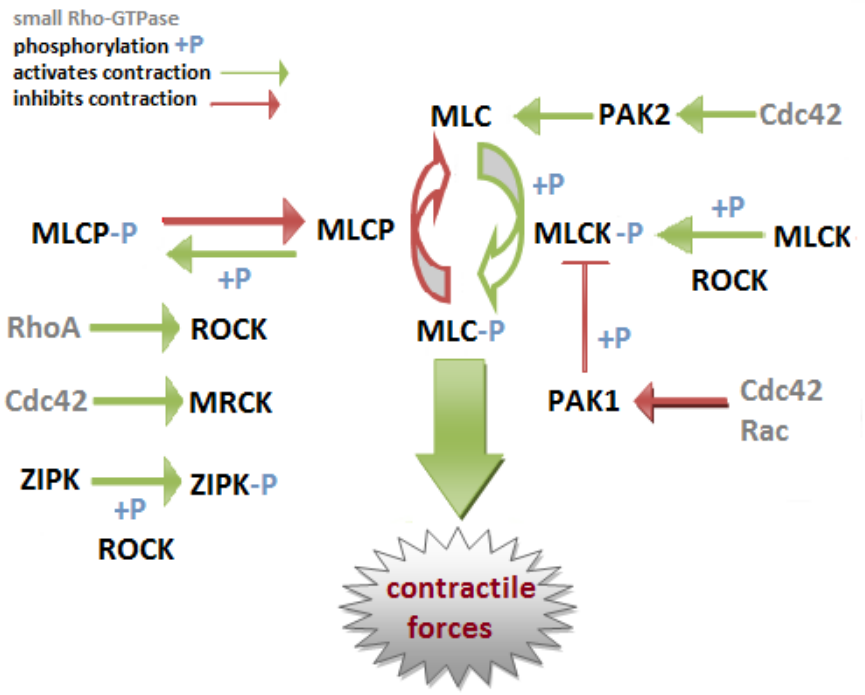


Figure IX: Interactions regulating contractile forces needed for amoeboid movement. Described in text.

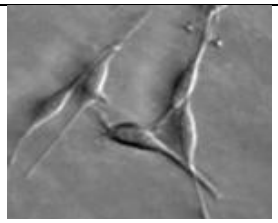
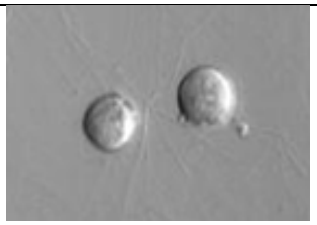
4.1.4. Mesenchymal and amoeboid invasion - the comparison

In cells invading in the mesenchymal mode polarization is easily observable. The front-rear arrangement is characterized by membrane protrusions and ruffling at the leading edge and a contracting rear. On the other hand, cells invading in an amoeboid manner do not have a morphologically easy distinguishable front. Although it has been observed that blebs form preferentially at the leading edge, the molecular mechanism is not yet clear. However, ROCK and ERM proteins have been found to be asymmetrically localized in the amoeboid cells, a possible mechanism of establishing front-rear polarity (Charras and Paluch, 2008; Paluch et al., 2005). Moreover, when transitions between blebs and lamelipodia were induced by switching on and off a photo-activatable version of Rac1, the new protrusion always formed at the same site, suggesting there is a common polarization mechanism for both lamellipodia and blebs (Bergert et al., 2012).

Further, it is eminent that either migration type depends on certain GTPases mediating signaling pathways involved in polarization and migration. The GTPases Rac and Cdc42 are important for mesenchymal migration, unlike amoeboid migration, which is primarily dependent on Rho mediated forces.

The most notable differences between the two types of migration are summed up below:

Figure X: The differences between mesenchymal and amoeboid invasion. Images from (Panková et al., 2010)

Invasion mode	Mesenchymal	Amoeboid
Morphology	elongated	rounded, apoptotic like
Observed structures	podosomes, invadopodia	membrane blebs
Adhesion	strong, numerous FA	weak, integrin independent
Force of movement	stress fibers at front and rear	cortical actin
Speed	0,1 -1 μm/min	up to 15 μm/min
GTPase	Rac, Cdc42	RhoA
Also utilized for	developmental processes	hematopoietic cells
Type of cancer (reference (Zijl et al., 2011))	fibrosarcoma, melanoma, breast cancer, lung cancer, prostate cancer	breast cancer, melanoma, lymphoma, sarcoma
Image of morphology		

Importantly, it is necessary to realize, that many invading cancer cell types are capable of migrating in both modes, often switching from one another according to the surrounding conditions.

4.2. Plasticity of cell invasion

The main reason why cancer is such a problematic disease is the plasticity of cell invasion and migration. Cells migrating in one invasion mode are often able to employ also another mode. Under specific conditions a certain migration manner is preferred, for example mesenchymal migration is preferentially used in stiffer matrices whereas more loose matrices allow amoeboid motility (DiMilla et al., 1991; Provenzano et al., 2008; reviewed in Brábek et al., 2010) . Importantly, blocking essential components of either invasion mode can lead to the switch to the second mode. This plasticity in cell movement complicates efficient cancer treatment.

4.2.1. EMT

EMT is short for epithelial to mesenchymal transition, the process during which a cell of epithelial phenotype and origin loses cell to cell contact, gains the ability to migrate and acquires mesenchymal characteristics. The transition can occur in an epithelial tissue cell, as well as in a cell from a collectively migrating cohort, and gives rise to an individually migrating cell. On the other hand, cells migrating individually in a mesenchymal manner can undergo an opposite process called mesenchymal to epithelial transition (MET), during which they lose motility, settle and retain cellular junctions. MET has been proposed as the primary mechanism of establishing a secondary tumor in tissues (Wells et al., 2008).

Interestingly, EMT is extensively studied not only in context of cancer cell invasion, but also in early tissue development, for example in gastrulation or neural crest formation (reviewed in Shook & Keller, 2003). The reason is apparent- both are regulated by a similar group of transcription factors like Wnt, Hedgehog or Notch.

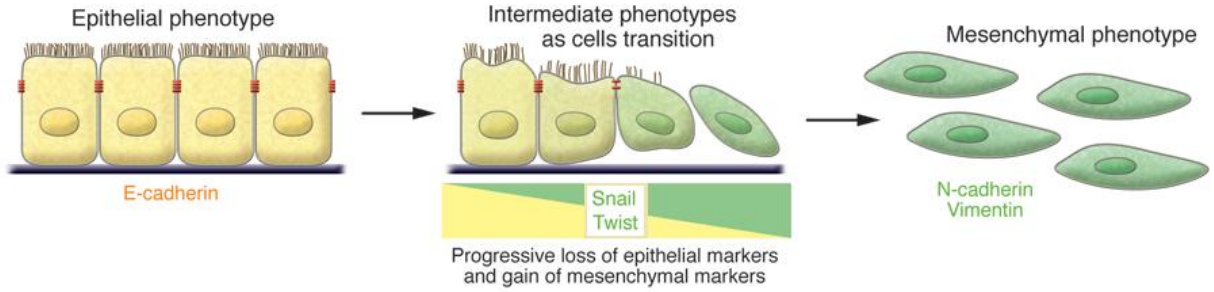


Figure XI: A simplified overview of EMT. Epithelial cells, expressing E-cadherin, lose their phenotype after activation of transription factor Snail and Twist and gain a mesenchymal phenotype typically accompanied by expression of N-cadherin and vimentin.. Adapted from (Kalluri and Weinberg, 2009) and edited.

As illustrated in figure XI, the main trigger of EMT is the activation of transcriptional factors such as Snai1, Twist or ZEB1 (Barberà et al., 2004; Yang et al., 2004; Aigner et al., 2010; respectively), which leads to the suppression of E-cadherin. This is compensated by the up-regulation of N-cadherins, typical for mesenchymal phenotype. The exchange of E- cadherin for N- cadherin is called "cadherin switch" and results in the loss of cell-cell contact (Umbas et al., 1994). Another hallmark of EMT is the expression of vimentin[5] and activation of pro-migratory signaling pathways through GTPases.

Since EMT increases tumor potential by enhancing individual cell invasion it is a possible therapeutic target. Researchers are testing blocking agents against Snail, Twist or cadherins. (Carneiro et al., 2013; Khan et al., 2013; Peinado and Cano, 2006).

[5] Vimentin is a cytoskeleton protein, a part of intermediate filaments type III and is expressed in mesenchymal cells. It has been utilized as a marker of mesenchymal cells and in elevated levels as a marker of colon cancer

4.2.2. AMT and MAT

Furthermore, as already mentioned, individually migrating cells can effectively change morphology to switch modes of invasion. The transition from amoeboid to mesenchymal is called AMT in short, equivalently MAT stands for mesenchymal to amoeboid.

Transitions often occur after blocking a critical component of invasion, for example blocking MMP triggers MAT (Wolf et al., 2003). Another factor leading to MAT is the weakening of cell-ECM adhesions by reducing the concentration of fibers in the ECM or activating the Rho signaling pathway (Sahai and Marshall, 2003). Apart from directly inducing MAT, the Rho/ROCK pathway can indirectly suppress the mesenchymal mode of invasion by activating ARHGAP22, a GAP of Rac, which inactivates Rac by promoting GTP hydrolyzation. By decreasing Rac activity, ARHGAP22 plays a role in amoeboid migration. Silencing it by siRNA induced a mesenchymal morphology in cells. A similar effect was observed after silencing DOCK3 and NEDD99, Rac activators. Treatment by siRNA against these proteins leads to increased MLC2 phosphorylation, which is typical for amoeboid motility (Sanz-Moreno et al., 2008).

Another pathway identified in regulation of mesenchymal to amoeboid transition is the degradation of RhoA mediated by Smurf1, a ubiquitin ligase. By regulating the amount of RhoA, Smurf1 plays an important role in cancer cell invasion. Silencing Smurf1 in mesenchymal colon cancer cells resulted in MAT and elevated migration levels (Sahai et al., 2007)

Moreover, the protein DIAPH3 (short for Diaphanous-related formin-3), also known as mDia2, was shown to be yet another regulator of transitions between amoeboid and mesenchymal invasion. DIAPH3 controls nucleation of filamentous actin by polymerization of F-actin and is proposed to be important in invadopodia (Lizárraga et al., 2009). Its loss is associated with rounded morphology, membrane blebbing and elevated levels of metastasis (Hager et al., 2012). Interestingly, the DIAPH3 gene lies in close proximity to the major tumor suppressor pRB on chromosome 13q in a locus, which is often deleted in cancers.

Yet another regulatory mechanism of cell migration is via the activation of LIM kinases (Lin11, Isl-1 and Mec-3 kinase) LIMK1 and LIMK2, which phosphorylate cofilin, an actin depolymerizing protein. Once phosphorylted, cofilin cannot induce actin depolymerization, leading to stabilized actin filaments. ROCK activates both LIMK1 (Ohashi, 2000) and LIMK2 (Sumi and Matsumoto, 1999), whereas Rac1 activates LIMK1 (Yang et al., 1998). In cells migrating in an mesenchymal manner the overexpression of LIMK induced a rounded, amoeboid morphology. However, the depletion of LIMK did not only suppress only amoeboid invasion, but also mesenchymal invasion. Therefore, LIMK plays a role in both amoeboid migration, where it contributes to acto-myosin contraction, but also in mesenchymal migration, where it supposedly influences lamellipodia formation (Mishima et al., 2010).

On the other hand, AMT is less documented. Recently, it was induced in cells naturally utilizing the amoeboid invasion after inhibiting the Rho pathway by silencing the glycoprotein NG2 (Paňková et al., 2012). A different study identified DOCK10 and Cdc42 to be closely related to the amoeboid phenotype by influencing MLC phosphorylation through MRCK. Melanoma cells, naturally invading in an amoeboid manner, lost their rounded morphology and switched to the elongated mesenchymal phenotype, after transfection with siRNA against DOCK10, a GEF specific for Cdc42 (Gadea et al., 2008).

Overall, the invasion mode is mostly dependent on the balance of GTPases, either associated with mesenchymal (Rac, Cdc42) or amoeboid (RhoA) invasion. Strengthening either pathway may lead to a transition of invasion mode (Sanz-Moreno et al., 2008).

4.2.3. CAT

When cells undergo a transition from collective invasion, they can either gain a mesenchymal phenotype during EMT, or switch to amoeboid movement in a process called collective to amoeboid transition, abbreviated CAT. During CAT cells dissociate from the migrating cohort by loosening β-integrin adhesion and gain amoeboid characteristics. Although CAT is the least common, it has been observed in melanoma (Hegerfeldt et al., 2002).

EMT, AMT, MAT and CAT all contribute to cell invasion plasticity by enabling reversible changes in cell migration and morphology. Figure XII summarizes their mutual relations and important factors related to the transitions.

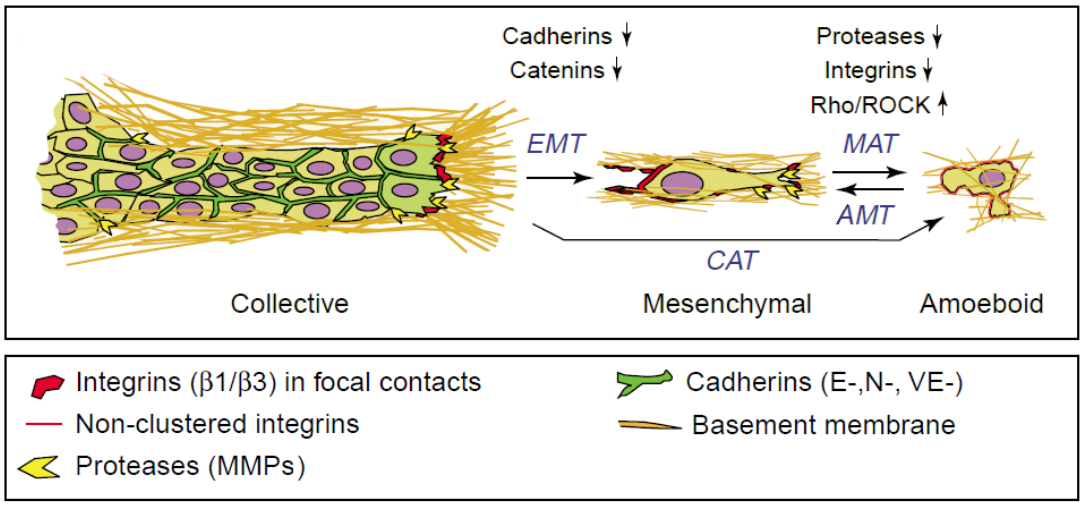


Figure XII: Illustration showing plasticity of cell invasion. Adapted from (Friedl, 2004).

4.3. Metastasis

Invasive cancer cell behavior results in metastasis. As shown in the previous chapter, cancer cell invasion, all collective, mesenchymal and amoeboid, demonstrates significant plasticity and is able to overcome many barriers. The process of invasion itself can trigger a cancer cell phenotype, observed in the case of EMT.

The course of metastasis was divided into several subsequent steps, called the metastatic cascade, that lead to the establishment of a new secondary tumor. The cascade is illustrated in figure XIII. First, the cell dissociates from the primary tumor, typically by regulating the expression of cadherins and catenins. Second, using either mode of invasion it travels through the basement membrane and surrounding ECM. The cell can penetrate a blood or lymph vessel wall in a process called intravasation, and flowing within, it spreads throughout the body. By adhering to the vessel wall it can leave the circulatory system (extravasation). Finally it colonizes the surrounding tissue, proliferates and establishes a secondary tumor, a metastasis. Throughout the last years, evidence has been gathered to support the assumption that the process of MET is essential during secondary tumor formation (reviewed in Gunasinghe et al., 2012).

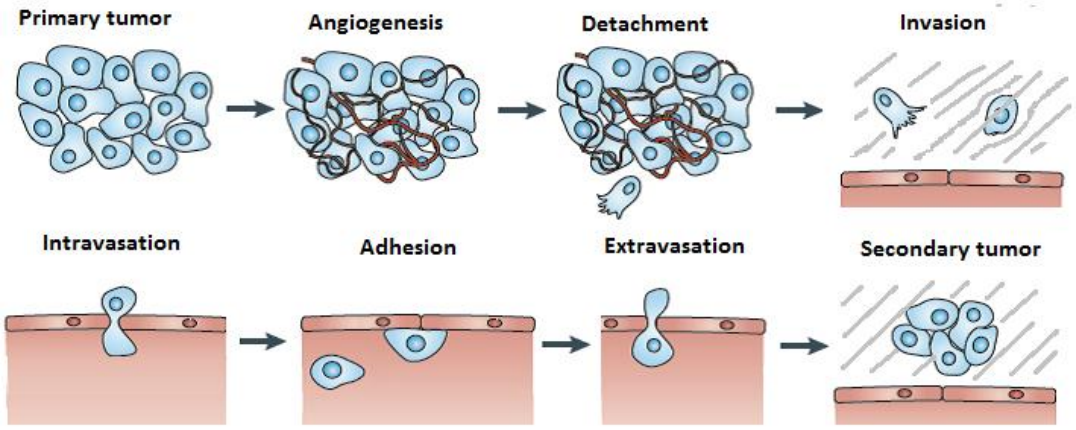


Figure XIII: Illustration of the metastatic cascade. Original picture was edited (Wirtz et al., 2011)

One of the main barriers tumors have to overcome is hypoxia, the shortage of oxygen. For such reason, tumors have gained the ability to induce the formation of new blood vessels, mainly by producing growth factors such as vascular endothelial growth factor A (VEGF) and platelet-derived growth factor (PDGF). The phenomenon is called angiogenesis. Since it is important for the survival of the primary tumor, it is sometimes considered to be the pre-initial step of the metastatic cascade (as in Brooks et al., 2010). Angiogenesis requires cell polarization and is another cancer process during which polarity proteins play an undoubted role.

5. Interplay of polarity proteins in cell invasion

Polarization is itself sufficient for directional movement, although highly persistent migration in one direction is possible due to extracellular signals. The cues that enhance directional migration can be of chemical basis in the case of chemotaxis, or can be physical such as the orientation and concentration of ECM fibers in case of durotaxis[6] (Lo et al., 2000). Overall, directionality of cell migration can be measured either as a ratio of the distance of the shortest path toward the cells actual path, or as a ratio of the shortest possible time to cover the distance toward the time the cell migrated (Pankov et al., 2005; reviewed in Petrie et al., 2009). Tumor cells can migrate in a polarized manner without any chemoattractant, persisting in a certain direction, which results in very efficient invasion through the ECM (Ridley et al., 2003).

The relation between Rho GTPases (Rac, Rho, Cdc42) and cell invasion has been stressed many times in this work as they are essential for setting front-rear polarity, for adhesion, for cytoskeleton regulation- all which play an important role in cell motility and invasion. Also the distribution of PI3K and PTEN regulates directional migration. Additionally, the cell polarity proteins complexes Scribble, Crumbs and Par also directly influence cell invasion by regulating polarity and cell junctions. In case of disruption of adhesions correct localization of polarity complexes is impaired. No wonder the interplay between polarity proteins, GTPases and cell invasion is intensively studied. So far, research has shed light on some very interesting signaling mechanisms which clarify the extensive mechanisms of cell motility with large implications for cancer cell invasion.

5.1. Links between Rho GTPases and cell polarity complexes

Largely interconnected in many signaling pathways, Cdc42 is proving to be a key factor in cell polarity. When it is activated, it can bind to Par6 that promotes the activity of aPKC (Joberty et al., 2000), which is necessary for polarized movement, correspondingly its inhibition results in random migration. Further, aPKC activation by Cdc42 is responsible for relocating the nucleus, Golgi and MTOC in migrating cells by phosphorylating glycogen synthase kinase-3β (GSK-3β), resulting in its inhibition. Under these conditions, adenomatous polyposis coli (APC) is stabilized at the leading edge and controls microtubule organization (Etienne-Manneville and Hall, 2003).

Rac activity is connected to polarity signaling primarily by its activator Tiam1, which is controlled by the Par proteins. In epithelial cells, Rac1, Par3 and Tiam1 are needed for the formation of tight junctions (Lin et al., 2000). Besides, it has been proposed that Par3 recruits Tiam1 to the leading edge

[6] Durotaxis is a phenomenon of preferential migration toward stiffer ECM. It also provides proof that cells actively sense the rigidity of their surroundings.

in migrating cells where it can activate Rac1 and initiate cell motility. Together the complex Par3-Tiam1 stabilizes front-rear polarization in migrating cells and permits directional migration (Pegtel et al., 2007).

Moreover, RhoA is also directly regulated by polarity proteins. Probably the most eminent is the regulation of RhoA at the leading edge by Cdc42-activated Par6 and its downstream effector Smurf1. Par6 recruits Smurf1 to the leading edge which then locally degrades RhoA, a regulation important for spatial localization of RhoA to the rear (Sahai et al., 2007; Wang et al., 2003). On the other hand, ROCK, the effector of RhoA, is able to phosphorylate Par3, which leads to the Par complex disruption and therefore deregulation of Tiam1, resulting in decreased Rac activation through Tiam1 (Nakayama et al., 2008).

The basolaterally localized Scribble complex can regulate Rho GTPases by the βPIX factor (Audebert et al., 2004), a GEF known to activate Rac1 and Cdc42. Interestingly, Scribble was shown to be essential in directional migration by co-localizing with Cdc42 and Rac to the cell front. Cells with Scribble knockdown were missing Cdcc42 and Rac at the leading edges, which led to impaired lamellipodia formation and, in effect, impaired directional migration (Dow et al., 2007). Furthermore, Scribble can bind to vimentin (earlier mentioned in the context of EMT) and this interaction protects Scribble from degradation, possibly enhancing directional migration after the transition to the mesenchymal phenotype (Phua et al., 2009).

5.2. Relation to EMT

Not only Scribble, but also the other two cell polarity complexes have an influence on EMT or other factors associated with it. Deregulation of cell polarity proteins leads to disrupted TJs and AJs and leads to loosened cell-cell contacts, a process typical for EMT. For example, Snail, one of the transcription factors able to activate EMT, represses the expression of Crumbs at gene level. This results in abolishment of Crumbs, but also Par from cell-cell junctions (Whiteman et al., 2008). Another process that links polarity proteins to EMT is the loss of E-cadherin. This was shown in cells lacking Scribble. They had impaired cell adhesions, did not form organized cell clusters and lost their epithelial morphology (Qin et al., 2005). The changes were caused by disrupted E-cadherin, providing evidence for the link between loss of E-cadherin and EMT. Other studies connect the polarity proteins with the TGFβ (transforming growth factor β) pathway, a key regulator of EMT (see figure XIV). TGFβ- mediated Par3 inhibition was shown to disrupt the Par complex leading to EMT (Wang et al., 2008). Further, the polarity protein Par6 was demonstrated to be a phosphorylation substrate of the TGFβ receptor type II (Ozdamar et al., 2005). Moreover, the study connects the TGFβ receptor mediated phosphorylation of Par6 to RhoA degradation by Smurf, which is activated by phosphorylated Par6.

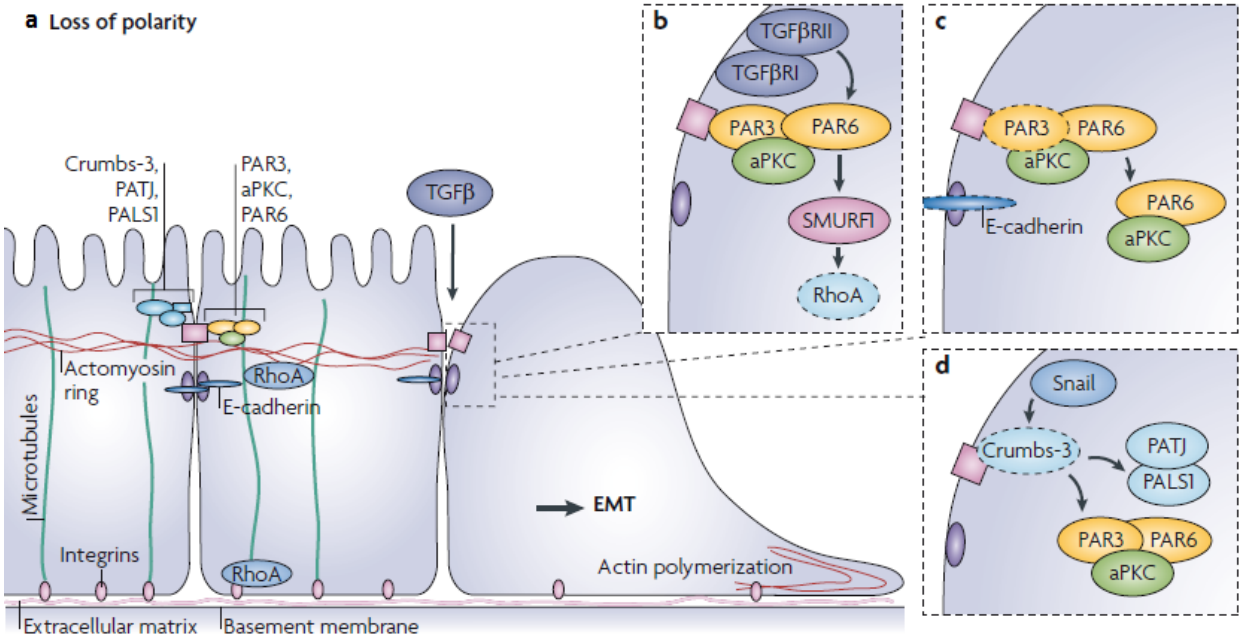


Figure XIV: Interaction of TGF during EMT (a): the phosphorylation of Par6 (b), inhibition of Crumbs (c) and inhibition of Par3 (d) Image adapted from (Iden and Collard, 2008)

5.3. Influence of phosphoinositides

There is a clear link between PIP3 and both Rac and Cdc42 at the leading edge. Activation of the GTPases by PIP3 was proved over 15 years ago (Keely et al., 1997), although at that time it was not clear what function it serves. Later it was shown that the accumulation of PIP3 provides a positive feedback for activation of Rac and Cdc42, but both differ in their precise role- Rac is necessary for forming a leading edge while Cdc42 is needed to maintain polarity (Srinivasan et al., 2003).

Rho GTPases further influence PTEN localization in cells. In migrating cells RhoA attracts PTEN to the rear. Interestingly, Cdc42 is also able to attract PTEN and localize it to the front membrane, however in a much weaker manner, therefore the RhoA regulation dominates. Notably, Rho was demonstrated to stimulate PTEN activity through ROCK, which enhances the polarization mechanism (Li et al., 2005).

5.4. Other significant interactions

Interestingly, two groups recently identified new pathways connected to Par3. One group investigated the down-regulation of Par3 in a breast cancer cell lines and showed, that invasiveness of cells positive for ErbB2 (also known as HER2) receptor was higher in cells with loss of Par3, probably due to an

indirect interaction between Par3 and ErbB2 (Xue et al., 2013). Correspondingly, the other group also found the decrease of Par3 to result in decreased latency of tumorigenesis in murine mammary gland cells with activated Ras or Notch pathways. The elevated tumor potential and invasive phenotype was caused by delocalization of aPKC and its activation of Stat3, which was accompanied by elevated MMP-9. (McCaffrey et al., 2012). Both studies established the role of Par3 as a tumor suppressor, but this seems to be individual for each cancer since Par3 over-expression in kidney and liver tumors correlated with poor patient outcome (Dugay and Goff, 2013; Jan et al., 2013).

It is of interest that not only Par3 is associated with elevated MMP levels. The complex aPKC-Par6 regulates levels of active aPKC, which correlated with levels of MMP-10 in non-small lung cancer. Blocking the kinase function aPKC diminished levels of MMP-10 proving a link between the two (Frederick et al., 2008)

In migrating epithelial cells, occludin, a tight junction protein, was shown to influence directional migration in a wound healing assay[7]. Occludin was proposed to activate PI3K at leading edge by localizating Par3 and aPKC through PATJ to the cell front (Du et al., 2010). The translocation of occludin was also confirmed in two breast cell lines (Martin et al., 2004), which suggests the mechanism of localization of polarity proteins might be regulated by a similar mechanism during both wound healing and cancer. However, some aggressive breast cancer cell lines are occludin negative. In the absence of occludin ZO-1, a binding partner of occludin at the tight junctions region, translocates to the cytoplasm, which results in expression of membrane type 1- MMP. Therefore ZO-1 contributes to tumor cell invasion. The combined results suggest that tight junction proteins are associated with other proteins to mediate cell invasiveness.

Altogether, the link between cell polarity proteins and cell migration has been the subject of intensive research from the time cell polarity complexes were identified. During the last two decades much progress has been made in claryfing the precise roles of individual proteins. This greatly encouraged further research of the mutual relations between cell polarity proteins and Rho GTPases leading to clarification of many regulatory mechanisms. However, the information we have today is still insufficient for perfect comprehension of cell motility at molecular level and many open questions remain concerning the role of polarity proteins in cell motility: What are their other binding partners? How can we modulate their localization in cells? What role do they play during MET? Why does their expression vary so much in various types of cancer? Hopefully, we will be able to answer most of them soon.

[7] A wound healing assay investigates directional migration by measuring the time/distance needed to close a scratch wound created in a cell monolayer

6. Therapeutic implications

In the previous chapters the link between cell polarity and cell invasion was depicted. From what was mentioned it is clear that polarity signaling is an adequate target for anti-metastatic drugs. By finding an effective way of regulating the initial course of cancer invasion, the course of metastasis could be inhibited.

Inhibitors of EMT and MMP were already mentioned in according chapters. Other studied targets for inhibition of cancer cell invasion include polarity proteins, Rho GTPases or proteins involved in their signaling pathways:

- Inhibition of prenylation of Rho GTPases by farnesyltransferase inhibitors were shown to regress tumor size already in the 1990's. Up till today it is a studied strategy of anti-metastatic therapy, although response rates are not so optimistic (reviewed in Holstein & Hohl, 2012).
- By regulating GEFs GAPs and GDIs it might be possible to modulate the function of Rho GTPases. Recently, a Rac-1 GEF inhibitor was shown to reduce metastasis in mice (Cardama and Comin, 2013).
- Since the PI3K pathway is often up-regulated in cancer, it is a potential cancer therapy target. Some of the inhibitors, such as XL147, have passed Phase II trials (Shapiro et al., 2014).
- Inhibiting MLCK by its specific inhibitor ML-7 resulted in increased cell apoptosis in a mouse mammary cancer cell line. What is more, it enhanced the effect of the anti-cancer drug etoposide[8] (Gu et al., 2006).
- Of the typical polarity proteins, the Par complex member aPKC is over-expressed or deregulated in many cancers. Inhibitors of the aPKC-Par6 interaction are emerging as potential anti-cancer therapeutics (Stallings-Mann et al., 2006).
- An interesting approach is to not block tumor dissemination, but instead promote MET. This was recently shown to be effective in melanoma (Pal et al., 2014).

However, the therapeutic significance of drugs against cancer cell invasion might be underestimated due to the Response Evaluation Criteria in Solid Tumors (RECIST) scoring system, commonly used to evaluate the drug efficacy. According to RECIST, favorable are those drugs that decrease the size of the tumor. Strikingly, the system doesn't rate the anti-metastatic impact. In effect, it might be time to reconsider the system and shift from anti-proliferative to anti-invasive strategies (Rösel et al., 2013; Weber, 2013).

Evaluating merely tumor size is even more misguiding in context of the today still more popular and accepted theory of cancer stem cells. This theory perceives a tumor as an "organ" based on the

[8] Etoposide is a clinically available anti-cancer drug, it induces apoptosis by blocking topoisomerase II.

findings of heterogeneous populations of cells inside the tumor. According to this statement, a certain group of cells in tumors identified by surface markers, was observed to be more capable of establishing metastasis. They were called metastatic cancer stem cells. Furthermore, these cells show elevated levels of invasion and are often resistant to drugs, becoming extremely dangerous in terms of malignant potential. To avoid the relapse of tumorigenesis, metastatic cancer stem cells should be the target of above mentioned therapeutic possibilities. Resemblance of EMT to de-differentiation gives rise to questions, whether EMT occurs predominantly in cancer stem cells. This can be measured by sorting cells in a population according to their surface markers. For example the phenotype CD44+/CD24- is a common marker of breast and prostate cancer stem cells. By analyzing cell invasion of prostate cancer cells, it was shown that only those with cancer stem cell traits where able to undergo EMT and invade (Klarmann et al., 2009). Another point of view, proposed among others by Robert Weinberg, one of the authors of the "Hallmarks of cancer", is that EMT gives rise to cells of mesenchymal morphology with stem-cell traits and higher invasion potential (Polyak and Weinberg, 2009). The phenomenon was shown in a population of breast cancer cells, which did not initially exhibit a cancer stem-like phenotype, but after induction of EMT proved to be CD44+/CD24- (Morel et al., 2008).

These findings still have to be precisely studied and validated in more cell lines in order to be widely accepted. However, it is now clear, that tumors are not clonal populations, but consist of more divergent populations, of which some might be more susceptible to EMT and invasion. The topic raises many questions about further directions in anti-cancer drugs.

7. Conclusion:

Cancer is still in the center of many scientists fields. By revealing molecular interactions and signaling pathways one after another, we are slowly coming closer towards revealing the nature of the disease. Unlike earlier, when genesis of primary tumors was considered to be the primary target, it is now generally accepted that blocking metastasis is necessary for better patient survival. However, this has proven to be a complex problem since cells are able to migrate in an collective, mesenchymal or amoeboid manner and inhibiting one invasion mode may promote another. The complexity of the whole issue is the main reason, why we lack an efficient treatment even after many decades of intensive research.

This work summarized the molecular mechanisms underlying each type of cancer cell invasion and transitions between them. Apart from that, it focused on the relation between invasion and polarity, reviewing their mutual interactions during polarization and directional migration. The cell polarity complexes Par, Scribble and Crumbs were put into context of Rho GTPase signaling. Additionally, I summarized the role of PIP3 and PIP2 in migrating cells. Finally, I briefly discussed the value of the proteins as therapeutic targets for cancer and metastasis.

In summary, cells are capable of generating forces needed for movement by remodeling the cytoskeleton and regulating cell polarity. Only after we fully understand the mechanism of cell motility will we be able to determine the best targets for blocking cancer cell invasion.

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