Prírodovedecká fakulta Univerzity Karlovej v Prahe

Katedra bunečnej biológie

The molecular mechanisms of transitions between mesenchymal and amoeboid invasiveness of tumor cells

Molekulárne mechanizmy prechodov medzi mezenchymálnym a améboidným spôsobom invazivity nádorových buniek

Bakalárska práca

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Praha 2009

Na tomto mieste by som sa chcela poďakovať predovšetkým svojim rodičom za ich podporu pri celom mojom štúdiu. Moja vďaka takisto patrí môjmu školiteľovi RNDr. Janovi Brábkovi, PhD. rovnako ako RNDr. Danielovi Röselovi, PhD. a všetkým členom mojej pracovnej skupiny za ich ochotu a trpezlivosť. Ďakujem svojmu múdrejšiemu bratovi Martinovi Paňkovi za pomoc pri grafickom spracovávaní obrázkov pre moju bakalársku prácu a taktiež aj kamarátke Magde Lokšovej za nezištné zapožičanie notebooku, keď sa mi ten môj v kritickej chvíli pokazil.

Prehlasujem, že som svoju bakalársku prácu na tému Molekulárne mechanizmy prechodov medzi mezenchymálnym a améboidným spôsobom invazivity nádorových buniek vypracovala sama, na základe uvedenej literatúry a konzultácií so svojím školiteľom.

Praha, 2009 Katarína Paňková

Abstrakt

Nádorové bunky vykazujú rôzne spôsoby migrácie pri invadovaní cez trojrozmerné prostredie. Invazivné stratégie jednotlivých nádorových buniek sa riadia podľa mezenchymálneho alebo améboidného modelu. Táto práca popisuje signálne dráhy podieľajúce sa na mezenchymálnom a améboidnom type motility nádorových buniek a zhŕňa molekulárne mechanizmy zapojené do tranzícií medzi nimi. Dôraz je kladený na signalizáciu Rho rodiny malých GTPáz – hlavne Rho, Rac a Cdc42 – ktoré regulujú procesy závislé na cytoskelete, o ktorých je známe, že sa zúčastňújú sa na bunečnom pohybe. Predpokladá sa, že početné interakcie medzi proteínmi z Rho rodiny a ich regulátormi a efektormi sú kľúčovými determinantmi konkrétneho spôsobu invazivity, ktorý bunky používajú. Mezenchymálna a améboidna invazivita vykazuje rozdielne adhezívne a proteolytické interakcie s okolitou matrix a zmeny ovplyvňujúce tieto interakcie môžu takisto viesť k tranzíciam.

Kľúčové slová:

nádorové bunky, invazivita, migrácia, mezenchymálny, améboidný, tranzícia, MAT, Rho GTPázy

Abstract

Tumor cells exhibit distinct modes of migration when invading through the three-dimensional environment. A single tumor cell strategy of invasiveness follows either mesenchymal or amoeboid pattern. This work outlines the signaling pathways involved in mesenchymal and amoeboid type of motility of tumor cells and summarizes the molecular mechanisms engaged in transitions between them. The focus is on signaling of Rho family of small GTPases – mainly Rho, Rac and Cdc42 - that regulate cytoskeleton-dependent processes taking place during the cell locomotion. Multiple interactions among the Rho family of proteins, their regulators and effectors are supposed to be the key determinants of a concrete type of invasiveness the cells are using. Mesenchymal and amoeboid invasiveness display different adhesive and proteolytical interactions with the surrounding matrix and the alterations influencing these interactions can also lead to the transitions.

Key words:

tumor cells, invasiveness, migration, mesenchymal, amoeboid, transition, MAT, Rho GTPases

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List of abbreviations

μm – micrometre

2D – two- dimensional

3D – three-dimensional

ATPase – adenosine nucleotide triphosphatase

ECM – extracellular matrix

F-actin – filamentous actin

FAK – focal adhesion kinase

FRET - fluorescence resonance energy transfer

GAP(s) – guanine nucleotide triphosphatase-activating protein(s)

GEF(s) - guanine nucleotide exchange factor(s)

GDP – guanine nucleotide diphosphate

GTP – guanine nucleotide triphosphate

GTPase – guanine nucleotide triphosphatase

MAT – mesenchymal-amoeboid transition or amoeboid-mesenchymal transition

MLC2 – myosin II light chain

MLCK – myosin light chain kinase

MLCP – myosin light chain phosphatase

MMP(s) – matrix metalloproteinase(s)

MRCK - myotonic dystrophy kinase-related Cdc42-binding kinase

MT(s) – microtubule(s)

N-WASP – neural Wiskott-Aldrich syndrome protein

P-MLC2 – phosphorylated myosin light chain 2

ROCK – Rho-associated serine-threonine protein kinase

WASP - Wiskott-Aldrich syndrome protein

WAVE - Wiskott-Aldrich syndrome protein family Verprolin-homologous protein.

1. Introduction

The ability of tumor cells to spread to distant locations in the body and to form metastases is the most life-threatening aspect of cancer. Therefore, understanding of the mechanisms underlying this feature is crucial. Metastasis is a complex process involving several steps: first of all, tumor cells must break away from the primary tumor and invade locally, through the barriers of the surrounding extracellular matrix (ECM) normally present in tissues. Further spreading of tumor cells to the secondary tumor sites occurs via the bloodstream or the lymph vessel system and therefore successfully metastasizing tumor cells must be capable of intravasation, survival in the bloodstream or lymphatic system and extravasation. In addition, tumor cells must be able to colonize the distant sites.

The local invasion to the adjacent tissue is one of the early steps in metastatic process and one of the key determinants of a metastatic potential of tumor cells. In order to overcome the ECM barriers, tumor cells develop different strategies. The collective migration of tumor cells, when cells retain their intercellular junctions, was observed on tumor cells migrating in groups or strands. This work describes single cell migration strategies of tumor cells, referred to as amoeboid and mesenchymal.

Amoeboid and mesenchymal type of invasiveness[1] are two phenotypes that are mutually interconvertible: abrogation or enhancement of the activity of specific molecular pathways that determine either of modes can cause a switch to the other type of invasiveness – this is called mesenchymal-amoeboid transition (MAT) or amoeboid-mesenchymal transition, respectively[2]. Transitions may play a role in the different stages of the metastatic process when a certain microenvironment requires phenotypical adaptation of the tumor cells. Up to date, several molecular mechanisms inducing MAT have been reported. Study of the mechanisms that are involved in the different types of invasiveness and that may possibly trigger MAT helps us to understand the plasticity in tumor cell migration strategies. This is important for the potential development of a cure for cancer that would efficiently suppress tumor cell invasiveness in the both modes.

[1] Term “invasiveness” can be defined as migration through the barriers. Terms “invasiveness” and “migration”, in case of the tumor cells can be used as synonyms because tumor cells in order to migrate overwhelm ECM barriers thus they invade when migrating.

[2] In this work, abbreviation MAT is used for the transitions in the either of directions.

2. An overview on single tumor cell invasiveness

The difference between two distinct types of single tumor cell invasiveness - the amoeboid and mesenchymal - is only obvious when observed in 3D environment. Two-dimensional assays such as wound healing often do not provide sufficient information on the concrete mode of invasiveness the cells are using. Moreover, a treatment that impairs tumor cell invasiveness in 2D environment does not necessarily influence the ability of cells to invade in 3D matrices (Sahai and Marshall 2003; Wolf *et al.*, 2003; Sahai *et al.,* 2007; Belletti *et al.,* 2008).

2.1 Mesenchymal type of invasiveness

The mesenchymal type of tumor cell migration can be compared to fibroblast – like motility. Except for fibroblasts, also keratinocytes, endothelial cells and some tumor cells use this pattern of migration. Cells with the mesenchymal type of motility have specific elongated spindle – like shape. In 3D matrices, cell are polarized, creating the obvious leading edge with the one or more leading pseudopods and the lagging cell body (containing nucleus, cytoplasm and organelles) that can be distinguished easily (see **Figure 1A**).

Translocation of the mesenchymally migrating cells begins with the formation of actin-rich filopodia and lamellipodia at the leading edge. This process is driven by the small GTPases from Rho family, primarily by Cdc42 and Rac (Ridley *et al.,* 1992; Nobes and Hall, 1995). Adhesive interactions with the ECM are present on the both cell poles and the contractile actin stress fibers attached to them generate the traction forces between the anterior and posterior cell edge (Scheetz *et al.,* 1998). Clustered integrins give rise to the focal adhesions that further recruit ECM-degrading proteolytical enzymes to perform pericellular ECM remodeling and to generate the path for migrating cells (Brooks *et al.,* 1996; Wei *et al.,* 1996). The velocity of migration in 3D matrices is approximately 0.1- 0.5 μm/min (Friedl *et al.,* 1998). The relatively slow speed of locomotion in mesenchymal integrin-dependent type of migration is determined by the necessity for the focal adhesions turnover during the translocation (Palecek *et al.,* 1997).

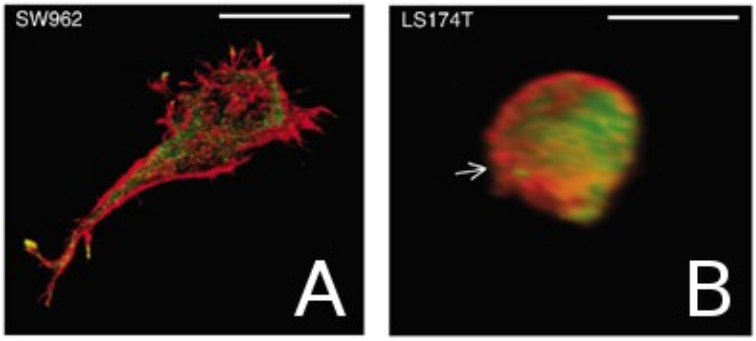

Figure 1 (adopted from Sahai and Marshall, 2003)**. Different morphologies of the invasive tumor cells.** The images are 3D software reconstruction from at least 20 confocal sections. The images were taken *in vitro* in a Matrigel[1] layer. Staining: F-actin (filamentous actin) in red by phalloidin and cortactin in green. Scale bars represent 20 μm. **A)** A squamous cell carcinoma cell line SW962 exhibits features characteristic for the mesenchymal type of invasiveness. Cell has elongated shape with the obvious leading protrusion. Attachments to the surrounding matrix can be seen. Actin is assembled into the long stress fibers, connecting the leading and the trailing edge of the cell, that are anchored to the membrane at the places of cell-ECM interactions. **B)** A human colon carcinoma cell line LS174T has the amoeboid-like phenotype with typical spherical cell morphology. Cortactin mediates the attachment of actomyosin to the plasma membrane. An arrow shows formation of F-actin rich blebs that are typical for the amoeboid-like invasiveness.

2.2 Amoeboid-like invasiveness

The amoeboid type of motility is named after the specific type of locomotion of amoeba *Dictyostelium Discoiduem*. *Dictyostelium* migrating strategy is characterized by the subsequent cycles of expansion and contraction of the cell body mediated by the cortically localized actin and myosin (Yumura *et al.,* 1984). Amoeboid-like movement was in higher eukaryotes described in leukocytes (Mandeville *et al.,* 1997; Friedl *et al.,* 2001) and the certain types of tumor cells (Sahai and Marshall 2003; Wolf *et al.,* 2003, Wyckoff *et al.,* 2006). Tumor cells migrating in the amoeboid pattern have in 3D substrata characteristic rounded shape. A typical morphology of a tumor cell with amoeboid-like invasiveness in 3D environment can be seen in **Figure 1B.**

The enhanced contractility of cells with amoeboid invasiveness, promoted by RhoA/ROCK signalization pathway (Sahai and Marshall, 2003; Wyckoff *et al.,* 2006), enables them to squeeze through the gaps in ECM fibers adapting their bodies to the pre-existing spaces

[1] Matrigel is a trade name of BD Biosciences product. It is a gelatinous mixture of ECM proteins (laminin, collagen IV, heparan sulfate proteoglycans) and growth factors produced by mouse sarcoma cell line.

(Mandeville *et al.,* 1997; Friedl *et al.,* 2001; Wolf *et al.,* 2003) and to eventually exert a sufficient force to deform surrounding ECM (Wyckoff *et al.,* 2006; Provenzano *et al.,* 2008). A tension maintained by cortical actomyosin results in membrane blebbing[1] of the amoeboid-like invading tumor cells that contribute to the cell motility (Keller and Eggli, 1998). Tumor cells with amoeboid migration move in 3D substrata independently on ECM degradation (Wolf *et al.*, 2003; Wyckoff *et al.,* 2006). The low-adhesion attachment to the substrate enables cells moving in the amoeboid pattern to translocate in 3D environment at relatively high velocities ranging from 2 μm/min observed on A375M2 melanoma cells (Sahai and Marshall, 2003) to 25 μm/min as is the peak migration velocity of lymphocytes in a collagen gel (Friedl *et al.,* 1994).

A summary of the concrete distinct characteristics of the mesenchymal and amoeboid type of invasiveness is outlined in the **Table 1.**

MESENCHYMAL		**AMOEBOID**
elongated	**morphology**	rounded
via integrin clusters creating the focal contacts and adhesions	**attachment to the ECM**	weak, short-term, integrins diffused in the membrane
ECM degradation, remodelling	**migration in the ECM**	proteolysis-indepedent pushing through the ECM
actin meshwork (leading edge), stress fibers (traversing the cell)	**organization of actin cytoskeleton**	contractile actin cortex
low	**velocity of the locomotion**	high
filopodia and lamellipodia	**cell membrane extensions**	intensive blebbing

Table 1. Comparison of the main phenotypic specifics of the mesenchymal and amoeboid pattern of invasiveness.

[1] It is necessary to mention that amoeba *Dictyostelium discoideum* is able to migrate using filopodia/lamellipodia strategy and membrane blebbing too, depending on the circumstances (Yoshida and Soldati, 2006). However, the tumor cell amoeboid-like migration is supposed to be Rac-induced lamellipodia formation independent (Sanz-Moreno *et al.,* 2008).

3. Mesenchymal invasiveness on the molecular level

A single tumor cell migration can be in general described in three subsequent steps: (1) the initial cell polarization and formation the leading protrusion that occurs in mesenchymal and amoeboid invasiveness by different mechanisms leads to (2) the interaction of the leading edge with ECM. Cell-ECM contacts trigger downstream signaling events that are followed by the (3) contraction of the rear of a cell and displacement of a cell.

Cell migration is a cytoskeleton-dependent process that is regulated by the Rho family of proteins. Rho family belongs to the Ras-superfamily of small GTP-binding proteins that act as molecular switches. The most intensively studied members of Rho family are Rac1, RhoA and Cdc42. Proteins of Rho family can be found in two forms: active, GTP-bound and inactive GDP-bound form. Switching between these two states is driven by several regulators. The guanine nucleotide exchange factors (GEFs) are proteins that mediate exchange of GDP for GTP and therefore activate Rho proteins so they can transduce signals to their downstream effectors as are protein kinases and adaptor-like proteins. Conversely, the GTPase activating proteins (GAPs) induce GTPase activity of Rho proteins what results in the hydrolysis of the bound GTP to GDP and subsequent inactivation of Rho proteins.

Mesenchymal migration begins with the extension of lamellipodia (flat two-dimensional protrusions containing branched network of the actin filaments) and filopodia (thin rod-like projections composed of the parallel actin fibers) at the cell edge. Rac and Cdc42 from the Rho family of proteins mediate actin polymerization by the regulation of WASP/WAVE[1] proteins (Miki *et al.,* 1998; Rohatgi *et al.,* 1999). The interaction of N-WASP and WAVE2 with Arp2/3 promotes nucleation of actin filaments and formation of the actin network at the leading edge (Suetsugu *et al.,* 2001). Cdc42 induces formation of filopodia (Nobes and Hall, 1995; Yang *et al.,* 2006) and affects the initial cell polarity by the regulation of microtubules (MTs) (Cau and Hall, 2005).

Lamellipodia and filopodia at the leading edge are stabilized by the interactions with ECM called focal contacts. The key components of the focal contacts are integrins, the

[1] Wiskott-Aldrich syndrome (WAS) is a rare X chromosome-linked disease characterized by eczema, thrombocytopenia and immunodeficiency. WASP is a protein the abnormal function of which in haematopoetic cells causes WAS symptoms. N-WASP was identified later in neural tissue. Despite its name, N-WASP is localized ubiquitously in the several tissue types. The abbreviation WAVE stands for **WA**SP family **Ve**rprolin-homologous protein.

transmembrane receptors that bind common components of ECM and mediate a mechanical linkage between ECM and actin cytoskeleton. Activation and co-clustering of integrins in the focal contacts is mediated by an adaptor protein talin that couples integrins with actin cytoskeleton (Calderwood *et al.,* 1999). Integrin clusters recruit via talin several adaptor proteins (e.g. paxilin, vinculin, zyxin) and signaling proteins (focal adhesion kinase - FAK, Src) and develop into the more stable focal adhesions (reviewed in Zeidel-Bar *et al.,* 2004). Attachment to the ECM is for the mesenchymally migrating cells crucial. Blocking of the β1 integrins in mesenchymally invading tumor cells was shown to lead to the loss of motility (Wolf *et al.,* 2003). Formation of the focal adhesions is associated with the Rho signaling-dependent rearrangement of the actin cytoskeleton to the long parallel actin stress fibers (Nobes and Hall, 1995). Distribution and the role of Rho GTPases in a mesenchymall migrating cell is depicted in the **Figure 2**.

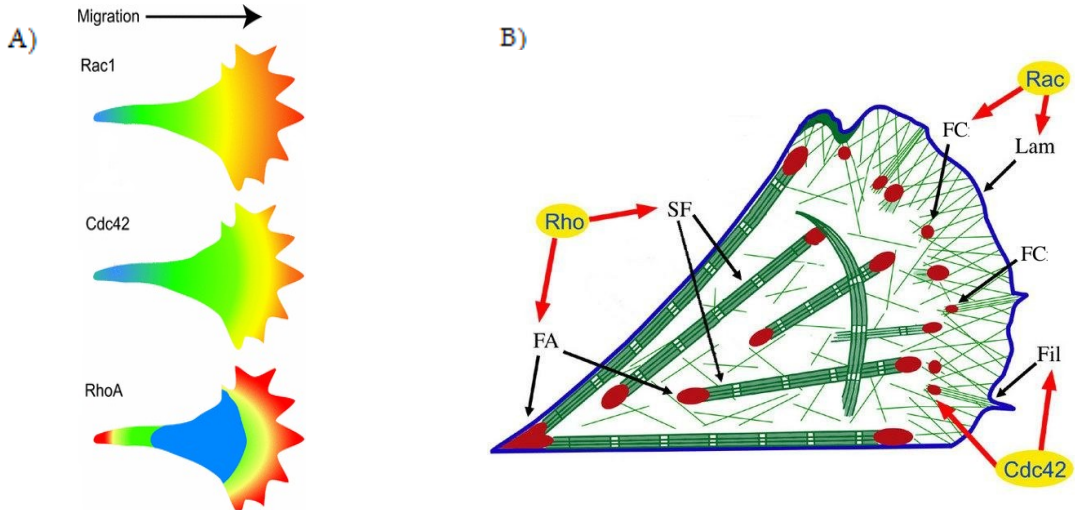

Figure 2. A) Localization of the activity of Rho GTPases (adopted from Kurokawa *et al.,* 2005). Distribution of the active Rho GTPases within a cell is visualized by the method of fluorescence resonance energy transfer (FRET). The activity of Rac1 and Cdc42 increases towards the leading edge. Cdc42 can only be found in its active form at the tips of the leading protrusions. RhoA localizes in the both cell poles at the focal adhesions in order to mediate contraction of actin stress fibers. **B) Contribution of the Rho family of proteins to the organization of actin cytoskeleton.** (adapted from the website of Vic Small laboratory, Austrian Academy of Sciences in Salzburg and Vienna http://cellix.imba.oeaw.ac.at/). At the leading edge, Rac signaling induces formation of lamellipodia (Lam) that are composed of actin meshwork. Small protrusions at the cell margin – filopodia (Fil) – are maintained by the activity of Cdc42. Rac- and Cdc42-mediated signalling induces formation of focal contacts (FC). Rho signaling leads to the development of focal contacts into the focal adhesion (FA). Active Rho is also necessary for the formation of long thick stress fibers (SF).

Clustered integrins recruit extracellular proteolytical enzymes as are matrix metalloproteinases (MMPs) and serine proteases (Brooks *et al.,* 1996; Wei *et al.,* 1996) to perform focalized pericellular remodelling of the ECM. Generation of the tube-like migratory pathways eases tumor cell migration.

The actual displacement of a cell depends on the traction force that arises from the contraction of stress fibers (Sheetz *et al.,* 1998). Contractility of the actin cytoskeleton is maintained mainly by Rho signaling. Rho in its "on" GTP-bound form activates its effector Rho-associated serine-threonine protein kinase (ROCK) that inactivates myosin-light-chain phosphatase (MLCP) (Kimura *et al.*, 1996). MLCP participates on the negative regulation of phosphorylation of myosin II light chain (MLC2) normally present in smooth muscle and non-muscle cells. An antagonistic effect has myosin-light-chain kinase (MLCK) that phosphorylates MLC2 and ROCK itself that was also found to directly phosphorylate MLC2 (Amano *et al.,* 1996). Once the MLC2 is phosphorylated (P-MLC2), the activity of myosin II ATPase is enhanced and myosin II promotes more efficient interactions with actin filaments what increases cell contractility. The other pathway involved in the regulation of a cell contractility involves Cdc42 signaling (Wilkinson *et al.,* 2005). Cdc42 acts through its downstream effector **m**yotonic dystrophy kinase-**r**elated **C**dc42-binding **k**inase (MRCK) (Leung *et al.,* 1998) and this signaling has an inhibitory effect on MLCP (Wilkinson *et al.,* 2005). The ratio of activities of MLC2 phosphorylating and P-MLC2 dephosphorylating regulators determines the resultant level of actomyosin contractility (schematically depicted in the **Figure 3**).

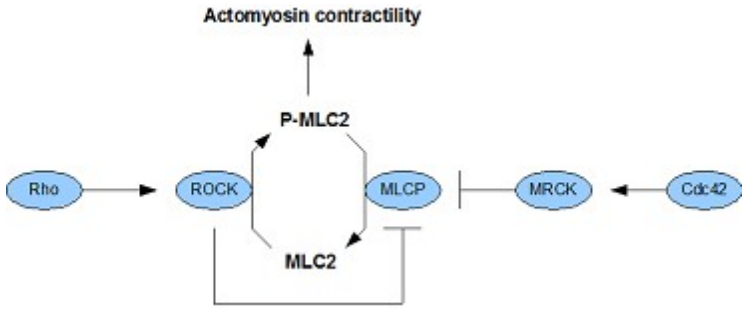

Figure 3. Regulation of MLC2 phosphorylation by Rho and Cdc42 and their effector kinases. Rho effector ROCK positively regulates actomyosin contractility: directly by the phosphorylation of MLC2 and indirectly through the inactivation of MLCP. Cdc42 increases P-MLC2 levels by the activation of MRCK that inhibits MLCP activity. (Not all of the regulators participating at the MLC2 phosphorylation are depicted.)

For the mesenchymal type of migration, Rho/ROCK signaling was shown to be dispensable (Sahai and Marshall, 2003). The activated Cdc42/MRCK pathway sufficiently compensates the loss of contractility in mesenchymally migrating cells after inhibition of Rho/ROCK pathway (Wilkinson *et al.,* 2005). Only the simultaneous abrogation of the function of both kinases, ROCK and MRCK, results in the significant decrease in cell motility of the mesenchymal BE colon carcinoma cells (Wilkinson *et al.,* 2005). One of the major differences between the mesenchymal and amoeboid invasiveness is the requirement for actomyosin contractility that is in mesenchymally migrating cells much lower (Sahai and Marshall, 2003; Wilkinson *et al.,* 2005; Wyckoff *et al.,* 2006). Both, the mesenchymal and amoeboid tumor cells, turn into the non-mobile phenotype when completely depleted of P-MLC2 mediated contractility (Wilkinson *et al.,* 2005).

Mesenchymally invading cells have to regulate disassembly of the focal adhesions at the trailing edge in order to detach from ECM and to translocate. The intracellular proteases calpains were shown to determine the stability of focal adhesions by the proteolysis of talin and further destabilization of the other components of focal adhesions (Franco *et al.,* 2004). Migration in the mesenchymal mode is significantly attenuated when the turnover of the adhesions is blocked by the inhibition of calpain 2 (Carragher *et al.,* 2006). The requirement of the regulated disassembly of focal adhesions is characteristic only for the mesenchymal migration (Carragher *et al.,* 2006). The regulation of stability of cell-ECM adhesions is also maintained by the MMPs and the composition of ECM (Belkin *et al.,* 2001).

4. Principles of the amoeboid-like motility of tumor cells

In the amoeboid-like moving cells, contractile cortical actomyosin network is crucial for the generation of the motive force (Yanai *et al.,* 1996). Submembranous actin is attached to the membrane by linking proteins from the 4.1 band superfamily – ezrin, radixin and moesin (reviewed in Niggli and Rossy, 2008). Rho/ROCK signaling maintained contractility of the membrane-attached actomyosin cortex (Sahai and Marshall, 2003) keeps the inside of a cell under a tension. As observed in *Amoeba proteus*, the intrinsic hydrostatic pressure can lead to the onset of a movement (Yanai *et al.,* 1996). The intracellular pressure results either in the detachment of actomyosin network from the membrane or rupture of the actomyosin cortex and the formation of spherical membrane herniations, so called membrane blebs (Keller and Eggli, 1998). The basic difference between the formation of lamellipodia in the mesenchymal motility and membrane blebbing is that the blebs are formed by the inflow of a cytoplasm (Keller and Eggli, 1998) thus the driving force of their formation is not the actin polymerization as it is in lamellipodia (Ridley *et al.,* 1992). Formation of the membrane blebs predominantly at the leading edge of the migrating cells (Keller and Eggli, 1998) may be triggered by the increased pressure on the membrane in a direction of the movement by the increased accumulation of contractile actomyosin at the posterior cortex as observed in *Dictyostelium* (Yumura *et al.,* 1984) or by the depletion of cortex-membrane linking proteins at the blebbing cell edge and the local dissasembly of actin (Keller and Eggli, 1998). Once the bleb is formed, actomyosin network under the membrane is restored (Charras *et al.,* 2006) and bleb is either retracted back or used for the further translocation (**Figure 4**).

The extension and stabilization of the bleb is proposed to be driven by the further inflow of the cytoplasm and the interactions with ECM. The exact nature of the cell-ECM attachments in amoeboid migration is a subject of the further research. In *Dictyostelium*, there are no integrins expressed and the substrate- binding forces are low (Friedl *et al.,* 2001). Similarly, in HT-1080 cell line after the induced transition to amoeboid migration, the loss of integrin clusters was observed (Wolf *et al.,* 2003). The independence of the amoeboid cancer cell migration on the β1 integrins was also reported (Hegerfeldt *et al.,* 2002). This, taken together with the impaired downstream signaling from α2β1 integrin complexes (Carragher *et al.,* 2006), implies reduced requirement for the formation of cell-ECM contacts in the amoeboid pattern of translocation.

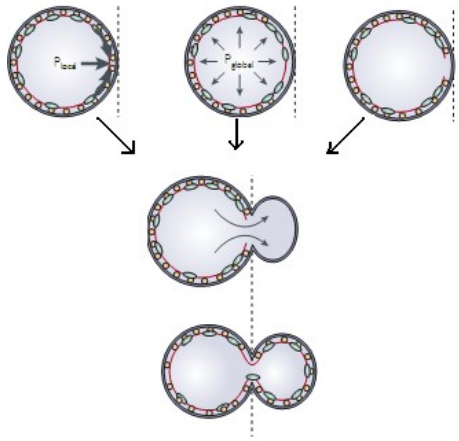

Figure 4 (adapted from Charras and Paluch, 2008). **Generation of the bleb.** Blebs can be generated either by the rupture of cortex by the increased local pressure on the membrane (upper left picture) or by the detachment of cortex from plasma membrane that occurs by the depletion of the linking proteins (upper middle picture) or by the local dissasembly of the cortex (upper right picture). The actin cortex under the bleb membrane is restored. Dashed lines depict the position of the original leading edge.

The specificity of amoeboid invasiveness of tumor cells is the independence on ECM proteolytic degradation (Wolf *et al.,* 2003). The migrating strategy of amoeboid cell lines in 3D substrata is based on Rho/ROCK maintained contractility (Sahai and Marshall, 2003; Wolf *et al.,* 2003; Wyckoff *et al.,* 2006). The proposed model of the amoeboid-like migration in 3D lattices is squeezing through the pre-existing spaces in ECM without remodeling of the surrounding matrix (**Figure 5**) (Sahai and Marshall, 2003; Wolf *et al.,* 2003). However, Rho/ ROCK maintained contractility was shown not only to maintain high cell deformability but also to exert sufficient forces to structurally change the ECM (Wyckoff *et al.,* 2006; Provenzano *et al.,* 2008).

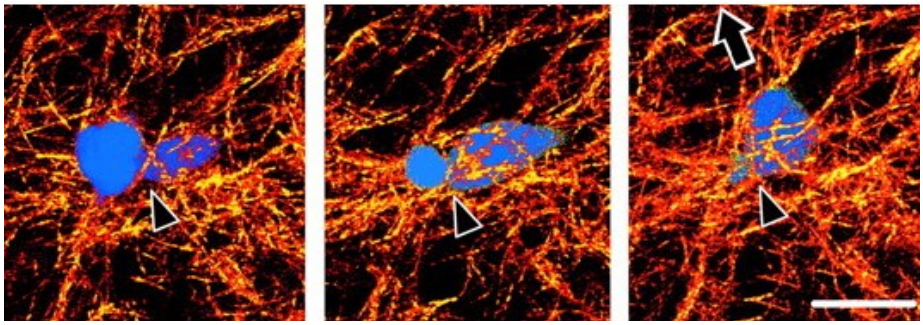

Figure 5 (adopted from Wolf *et al.,* 2003). **Propulsive movement of the amoeboid-like moving tumor cell in 3D collagen I gel.** The arrowheads show the constriction ring, a morphological adaptation that cell uses for squeezing through the narrow gaps. The amoeboid movement in HT-1080 cell line was induced by the inhibition of pericellular proteolysis.

5. Molecular mechanisms underlying MAT

5.1 Amoeboid to mesenchymal transitions by affecting the actomyosin contractility

As described previously, amoeboid invasiveness has a strong requirement for actomyosin contractility maintained by Rho/ROCK signaling (Sahai and Marshall, 2003; Wyckoff *et al.,* 2006). Silencing of the Rho/ROCK pathway in amoeboid tumor cells was the first observed mechanism that induces transition from amoeboid to mesenchymal invasiveness (Sahai and Marshall, 2003). The inactivation of either Rho or ROCK in A375M2 melanoma cells with the blebbing amoeboid-like phenotype results in the transition to the mesenchymal-like morphology (Sahai and Marshall, 2003). Amoeboid and mesenchymal migration were shown to exhibit different susceptibility to the treatment inhibiting cell contractility. The amoeboid line A375M2 switches after the low doses of blebbistatin, a myosin II ATPase inhibitor, to the elongated mesenchymal phenotype of invasiveness whereas mesenchymally migrating BE colon carcinoma cell line shows significantly impaired ability to invade after the same treatment (Wilkinson *et al.,* 2005). These findings together indicate that the increased level of P-MLC2 that mediates cell contractility can be considered as an indicator of amoeboid-like motility. Rho/ROCK signaling not only drives the amoeboid migration but also impairs Rac signaling by activating GAP of Rac called ARHGAP22; thereby Rho signalling inhibits the mesenchymal pattern of invasiveness (Sanz-Moreno *et al.,* 2008).

Active Cdc42 was also found necessary for maintenance of the amoeboid pattern of invasiveness (Gadea *et al.,* 2008). A Cdc42 GEF named DOCK10 activates Cdc42 and its effectors N-WASP and Pak2 kinase (Gadea *et al.,* 2008). DOCK3 and N-WASP are supposed to act in a complex (Gadea *et al.,* 2008). Inhibition of either DOCK10, N-WASP or Pak2 in amoeboid A375M2 melanoma cells leads to the transition to mesenchymal-like invasiveness (Gadea *et al.,* 2008). The proposed way of action of DOCK3 and Pak2 is by decreasing the levels of P-MLC2 (Gadea *et al.,* 2008). N-WASP is known to activate Arp2/3 complex (Suetsugu *et al.,* 2001) but the mechanism of the contribution of Arp2/3 to the amoeboid invasiveness is unclear. Alteration of the other signalization pathway from Cdc42 through MRCK leading to the increased levels of P-MLC2 was not proved to trigger transition (Wilkinson *et al.,* 2005). The interactions among the Rho GTPases and their involvement in MAT (see also **5.2**) are depicted in the **Figure 6**.

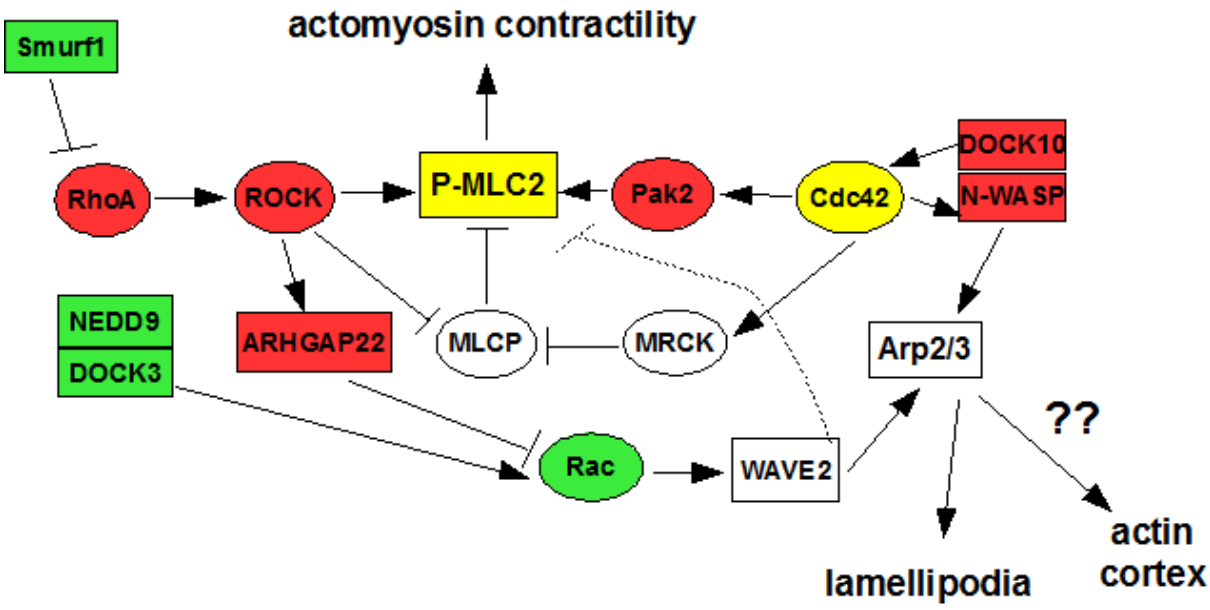

Figure 6. Interactions among the components of signaling pathways involved in transitions. An inhibition of activity of the proteins highlighted in red was shown to trigger amoeboid to mesenchymal transitions. Abrogation of the function of the proteins depicted in green color induces conversion from mesenchymal to amoeboid mode of invasiveness. Components that are necessary for the invasiveness in the both modes (lead to a non-mobile phenotype if not present) are picked out in yellow. A dashed line from WAVE2 to P-MLC2 implies unknown mechanism of the inhibition of P-MLC2 by WAVE2. For the citations, see the text (chapters **4.**, **5.1** and **5.2**).

5.2 MAT induced by inhibition of the initial cell polarization

Rac is believed to be the key regulator of the lamellipodia formation in the mesenchymally migrating cells (Ridley *et al.,* 1992; Miki *et al.,* 1998). Inactivation of Rac induces rounded phenotype and increased levels of P-MLC2 (Sanz-Moreno *et al.,* 2008). The same effect has the inactivation of either NEDD9 or DOCK3 that mediate the activation of Rac (DOCK3 is a GEF of Rac, NEDD9 is an adaptor protein creating the complex with DOCK3) (Sanz-Moreno *et al.,* 2008). Rac-dependence for the mesenchymal pattern of invasiveness is not only restricted to the lamellipodia formation. Rac1 also influences focal adhesions assembly and subsequent stress fibers formation (Guo *et al.,* 2006) that are important for the mesenchymal type of invasiveness. Moreover, Rac supresses cell contactility through its efector WAVE2 that negatively regulates phosphorylation of MLC2 and therefore unfavors the amoeboid pattern of motility (Sanz-Moreno *et al.,* 2008). Thus, Rac signalization can be considered to be crucial for the mesenchymal migration of tumor cells.

Local inhibition of cell contractility at the leading edge is necessary for the efficient formation of the leading protrusions in the mesenchymal invasiveness. Depletion of RhoA at the leading edge is maintained by Smurf1 protein (an E3-ubiquitin ligase[1]) that targets RhoA for the proteolytical degradation (Wang *et al.,* 2006) and therefore locally impairs Rho/ROCK signaling. Smurf1 favors the mesenchymal type of invasiveness and is believed to be an important determinator of this type of migration considering the fact that inhibition of Smurf1 itself in mesenchymal BE colon carcinoma cells is sufficient to induce transition to the amoeboid invasiveness (Sahai *et al.*, 2007). Interactions among the components of the Rho family signaling pathways involved in transitions can be seen in the **Figure 6**.

Cdc42 is involved in multiple pathways crucially regulating cell migration and therefore the inactivation of Cdc42 leads to the non-mobile phenotype (Gadea *et al.,* 2008). In mouse embryonic fibroblasts, formation of the Cdc42 induced filopodia is negatively regulated by p53, a transcription factor that functions as tumor suppressor (Gadea *et al.,* 2002). The concrete mechanisms of this interference is not known but it is proposed that p53 acts downstream from Cdc42 (Gadea *et al.,* 2002). The inactivation of p53 in mouse embryonic fibroblasts in 2D leads to the constitutive formation of the filopodia (Gadea *et al.,* 2002) whereas in mesenchymally migrating A375P melanoma cells in 3D environment, the abrogation of p53 function was strikingly found to induce transition to the amoeboid phenotype with the rounded morphology, increased RhoA activity and membrane blebbing (Gadea *et al.,* 2007).

Cdc42 is also a key regulator of the cell polarity in fibroblast migration that is maintained by the MT cytoskeleton (Cau and Hall, 2005). The dynamics of MTs in cell is regulated by several stabilizing and destabilizing proteins that interact either with the tubulin subunits or polymerized MTs. One of MT regulating proteins is stathmin (also known as Op18) that contributes to the MT destabilization (Curmi *et al.,* 1999). It was observed on the HT-1080 fibrosarcoma cells that stathmin 1 (a stathmin isoform) expression enhances migration in 3D matrices, albeit does not influence the invasiveness in 2D environment (Beletti *et al.,* 2008). The enhanced migration of the originally mesenchymally invading HT-1080 cells in 3D assays is coupled with the gain of rounded shape (Beletti *et al.,* 2008) what suggests that stathmin 1 may contribute to MAT. The cell morphology was the only sign of the amoeboid

[1] Ubiquitination is performed by the complex of three enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligase complex. E3 complex acts specifically and is responsible for targeting the particular protein for the degradation.

invasiveness that Belleti's group observed (2008), there is no evidence yet on stathmin 1 (over)expression - induced changes in the cell surface integrins distribution, increased P-MLC2 levels nor the membrane blebbing. The MT-destabilizing activity of stathmin is negatively regulated by the phosphorylation (Curmi *et al.,* 1999). The phosphorylation was shown to be triggered by the cell adhesion to the fibronectin component of the ECM substrata; therefore stathmin 1 influences an adhesion-dependent MT polymerization (Belletti *et al.,* 2008).

MTs are important for the maintenance of the cell polarity in mesenchymally migrating cells (Cau and Hall, 2005) but in amoeboid-like migration of neutrophils (Mandeville *et al.,* 1997) the role of MTs is opposite. There is an evidence that in neutrophils, a treatment inducing MT disassembly supports the motility of neutrophils by selective activation of ROCK and ROCK – induced increased levels of P-MLC2 (Niggli, 2003). These findings support a hypothesis that the mesenchymal and amoeboid invasiveness have a different requirement for the MT cytoskeleton maintained cell polarity. Disruption of MTs appears to be an appropriate signal leading to MAT (Beletti *et al.,* 2008) however; the role of destabilization of MTs in amoeboid-like tumor cells has not been reported yet.

5.3 Proteolytical, adhesive and remodeling interactions with the ECM and MAT

The increased proteolysis of ECM by tumor cells expressing extracellular matrix metalloproteinases (MMPs), serine proteases and cathepsins was considered to be the crucial determinant of the tumor cell invasiveness until the blocking of the extracellular proteolysis revealed extracellular proteases-independent mode of invasiveness (Wolf *et al.,* 2003). Originally mesenchymally migrating HT-1080 fibrosarcoma cells are able to invade in 3D collagen lattices after the treatment with inhibitory cocktail in ECM proteolysis – independent manner, showing the typical features of the amoeboid invasiveness as is rounded morphology, integrin diffusion in plasma membrane and the use of cortical actomyosin contractility during the migration (Wolf *et al.,* 2003; Carragher *et al.,* 2006, Wyckoff *et al.,* 2006). The induced amoeboid migration of HT-1080 cells after the inhibition of proteolysis is associated with the decreased cell surface expression of $\alpha 2\beta 1$ integrins and reduced level of phosphorylated FAK (Carragher *et al.,* 2006) suggesting decreased requirement for the formation and signaling from focal adhesions. Although the amoeboid invasiveness is supposed to be $\beta 1$ integrin-independent (Hegerfeldt *et al.,* 2002), suppression of the $\beta 1$ integrin-mediated adhesion to the

ECM in mesenchymally migrating cells was not shown to be a sufficient mechanism for the induction of MAT (Carragher *et al.,* 2006).

A recent study showed how the tumor cells are able to switch between the Rho/ROCK dependent and Rho/ROCK-independent mode of invasiveness with respect to the spatial organization of the surrounding collagen fibers (Provenzano *et al.,* 2008). In mesenchymally migrating MDA-MB-231 breast carcinoma cells the Rho/ROCK independent migration can only be used when the collagen fibers are pre-aligned in a parallel way (Provenzano *et al.,* 2008). Rho/ROCK mediated contractility of the mesenchymal MDA-MB-231 cells is used for the active reorganization of the filaments in the matrices where the filaments are not pre-aligned (Provenzano *et al.,* 2008).

6. Conclusions and discussion

The alterations in signaling involved in single cell migration may trigger a conversion from the mesenchymal pattern of migration to the amoeboid and vice versa. The crucial pathways engaged in transitions can be divided in three groups.

1) Signaling influencing the cell contractility. Increase in P-MLC2 is coupled with amoeboid invasiveness (Sahai and Marshall, 2003; Wyckoff *et al.,* 2006) whereas impaired Rho/ROCK signalization and decreased P-MLC2 level leading to the impaired contractility is a feature of the mesenchymal invasiveness (Wilkinson *et al.,* 2005; Sanz-Moreno *et al.,* 2008). The treatment influencing P-MLC2 levels was shown to reversibly change the type of invasiveness (Sahai and Marshall, 2003; Sanz-Moreno *et al.,* 2008). To fully understand this mechanism, it is necessary to elucidate the pathways that maintain the residual contractility after the inhibition of the Rho/ROCK signaling and to determine the regulators of these pathways.

2) Signaling influencing onset of the leading extension. The inhibition of the formation of lamellipodia by the abrogation of Rac signaling is a crucial mechanism leading to the transition from the mesenchymal to amoeboid invasiveness (Sanz-Moreno *et al.,* 2008). Moreover, there is an evidence of a crosstalk between the Rho and Rac signaling (Sanz-Moreno *et al.,* 2008) that is believed to determine the mode of migration. The role of MTs in the transitions has emerged just recently (Belletti *et al.,* 2008) and remains to be described in more details. Cdc42 is a component of the signaling pathways that deserves a special interest because of the wide range of processes leading to the cell migration in the both modes that depend on the Cdc42 mediated signaling of migration (Nobes and Hall, 1995; Cau and Hall, 2005; Gadea *et al.,* 2002; 2007; 2008; Yang *et al.,* 2006).

3) Signaling arising from the interactions with ECM. Inhibition of the pericellular proteolysis induces transition to amoeboid invasiveness by triggering a complex response in the cell (Wolf *et al.,* 2003) but the molecular mechanisms underlying this process are still not described. There is not much known about the signaling events that lead from the inhibition of proteolysis to the reorganization of the actin cytoskeleton, loss of adhesion-dependent motility and others specifics of the amoeboid-like locomotion. There is an evidence that tumor cells can „sense“ the surrounding environment and adapt the mode of invasiveness they are using

to the organization of the ECM (Provenzano *et al.,* 2008). However, it has been not proposed yet how this is possible and which pathways are involved. The summary of the reported transitions from the mesenchymal to amoeboid invasiveness can be seen in the **Table 2**.

The definition of the concrete type of movement the cells are using after a transition is problematic. In the most of the reported transitions, the feature determining the transition from mesenchymal to amoeboid invasiveness is the spherical cell morphology. There is usually some information on the elevated P-MLC2 levels or the actin localization and related features as are the increased RhoA activity or membrane blebbing, but the other specifics as are the integrin diffusion within the cell membrane or the use of MMPs are not stated. The similar situation is in the documentation of the amoeboid to mesenchymal transitions. The issue is that it is not know whether all the transitions observed are „complete“ or whether there is a possibility that a specific treatment can trigger „partial“ transitions where the resultant phenotype is a mixture of the mesenchymal and amoeboid characteristics. Therefore, the exact definitions for determining the transitions should be set.

There is a striking evidence that the mesenchymal to amoeboid transitions observed in 3D collagen I gels might be an experimental artifact (Sabeh *et al.,* 2009). It is also suggested that the amoeboid invasiveness of tumor cells observed *in vivo* can only occur under specific conditions and is not a common and widespread alternative of the single tumor cell migration (Sabeh *et al.,* 2009) as was thought previously (Sahai and Marshall, 2003; Wolf *et al.,* 2003). Therefore, further research in this area is necessary to determine whether the phenomenon of MAT does even occur *in vivo*.

Treatment	Tumor cell line	Changes observed in 2D environment		Changes observed in 3D environment				Other characteristics of the induced amoeboid phenotype	Citation
		Spherical shape	Ability to migrate	*In vitro* assay used	Spherical shape	Ability to migrate	Amoeboid migration observed *in vivo*		
↓ ECM proteolysis	HT-1080	Not stated	Not stated	Collagen I gel	Yes	Not changed	Dissemination within the dermis	Cortical actin, constriction rings, diffused integrins	Wolf *et al.*, 2003
↓ Rac signalling	A375M2 WM1361	Yes	Not changed	Collagen I gel	Yes	Not stated	Subcutaneous dissemination, colonization of the lungs	↑ P-MLC2	Sanz- Moreno *et al.*, 2008
↑ stathmin 2 (↓MT stability)	HT-1080	Yes	Not changed	Collagen I gel Matrigel	Yes	Increased	Colonization of the lungs	Not stated	Belletti *et al.*, 2008
↓ Smurf1 (↑Rho degradation)	MDA-MB-231 BE	Yes	Impaired	Matrigel	Yes	Not stated	Subcutaneous dissemination	Cortically localized actin, membrane blebbing	Sahai *et al.*, 2007
↓ p53	A375P	Not stated	Not stated	Matrigel	Yes	Increased	Not stated	RhoA activation, membrane blebbing	Gadea *et al.*, 2007

Table 2. Reported transitions from the mesenchymal to amoeboid invasiveness.

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