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Molecular mechanisms of amoeboid invasion of cancer cells

PhD thesis

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I hereby declare that this submission has been developed independently, only based on my the best knowledge and studying cited literature and consultation with my supervisor. This work is also not part of any previously neither written nor published material.

Prague, August 2012 Daniela Paňková

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LIST OF ABREVATIONS

A337/377RP (A3)	highly metastatic cells
AMT	amoeboid-mesenchymal transition
ECM	extracelullar matrix
EMT	epithelial-mesenchymal transition
FITC	fluorescein isothiocyanate
LW13K2 (K2)	parental non-metastatic fibroblast-like cells
MAT	mesenchymal-amoeboid transition
MMP	matrix metalloprotease
MT-MMP	membrane-bound metalloproteases
TIMP	tissue inhibitors of metalloproteinases
2D	two dimensional
3D	three dimensional
uPA	urokinase-type plasminogen activator
TPA	12-*O*-tetradecanoylphorbol-13-acetate
WASP	Wiskott-Aldrich Syndrome protein

SUMMARY

Tumour cell invasion is one of the most critical steps in malignant progression. It includes a broad spectrum of mechanisms, including both individual and collective cell migration, which enables them to spread towards adjacent tissue, and form new metastases. Understanding the mechanisms of cell spreading, and invasion, is crucial for effective anticancer therapy. Two modes of individual migration of tumour cells have been established in a three-dimensional environment. Mesenchymally migrating cells use proteases to cleave collagen bundles, and thus overcome the ECM barriers. Recently described protease-independent amoeboid mode of invasion has been discovered in studies of cancer cells with protease inhibitors.

During my PhD study, I have focused on determining the molecular mechanisms involved in amoeboid invasion of tumour cells. We have examined invasive abilities in non-metastatic K2 and highly metastatic A3 rat sarcoma cell lines. We have shown that even though highly metastatic A3 rat sarcoma cells are of mesenchymal origin, they have upregulated Rho/ROCK signalling pathway. Moreover, A3 cells generate actomyosin-based mechanical forces at their leading edges to physically squeeze through the collagen fibrils by adopting an amoeboid phenotype.

Amoeboid invasiveness is also less dependent on integrin adhesion to the extracellular matrix, and we have suggested the potential role of NG2 proteoglycan in amoeboid cell adhesion and invasion. We found that NG2 knockdown leads to decreased invasion, as well as reduced level of active Rho in both highly metastatic A3 rat sarcoma and A375M2 human melanoma cells. Conversely, overexpression of NG2 proteoglycan correlated with elevated Rho-GTP expression, and increased invasiveness of non-metastatic K2 rat sarcoma cells. Our findings strongly suggest the role of NG2 proteoglycan as an adhesive molecule involved in amoeboid invasion as well as its potential connection to the Rho/ROCK signalling pathway.

We have also compared invasive properties of G3-EM parental primary breast cancer cells and its derived neoplastic transformed G3S1 line. Our results have shown elevated cytoskeletal dynamics in more invasive G3S1 cells.

SUMMARY IN CZECH

Invasivita nádorových buněk je jedním z nejdůležitějších kroků v procesu maligní progrese. Je charakterizovaná širokým spektrem mechanismů, které zahrnují jak individuální, tak kolektivní migrací buněk. Během procesu invasivity se nádorové buňky šíří do okolních tkání, kde zakládají nové metastázy. I proto je pochopení mechanismů buněčné invasivity zásadním krokem k účinné protinádorové terapii. Individuálně migrující buňky vykazují ve 3D prostředí dva způsoby invasivity. Mesenchymalně invadující buňky produkují během své migrace proteázy, které využívají k degradaci kolagenových vláken. Studie srůznými typy nádorových buněk zaměřené na inhibici proteáz poukázaly na nový amoeboidní mechanizmus invasivity buněk, který není závislý na degradací extracelulární matrix pomocí proteáz.

Během mé disertační práce jsem se zaměřila na studium molekulárních mechanismů uplatňujících se v amoeboidní invasivitě nádorových buněk. Pro naše experimenty jsme využili potkaní sarkomový model sestávající z parentální nemetastazující linie K2 a od ní odvozené vysoce invasivní linie A3. Ukázali jsme, že i když je vysoce metastatická sarkomová linie A3 buněk mesenchymálního původu, její invasivní vlastnosti jsou závislé na upregulaci Rho / ROCK signální dráhy, která vede ke generování protrusivních sil na vedoucích okrajích amoeboidně migrujících buněk.

Amoeboidní invasivita je málo závislá na integrínové buněčné adhezi k extracelulární matrix, a proto byl náš další výzkum zaměřený na úlohu NG2 proteoglykanu jako molekuly schopné zprostředkovat adhezi nezávisle na integrínech. Zjistili jsme, že umlčení NG2 prostřednictvím siRNA vede k poklesu invasivity a k snížení aktivity Rho GTPásy jak u potkaní, vysoce metastatické linie A3, tak i u lidské melanomové linie A375M2. Naopak, overexprese NG2 proteoglykanu korelovala se zvýšenou hladinou Rho-GTP a také se zvýšeným invasivním potenciálem u nemetastatické, parentální potkaní sarkomové linie K2. Tyto naše výsledky naznačují možnost uplatnění NG2 proteoglykanu jako adhesivní molekuly, která se podílí na amoeboidní invasivitě, stejně jako její propojení na Rho/ROCK signální dráhu.

Dále jsme srovnávali vlastnosti G3-EM linie primárního karcinomu prsu a jeho odvozené transformované G3S1 linie. Zjistili jsme, že neoplasticky transformovaná invazivnější G3S1 linie vykazuje oproti méně invasivnější G3-EM linii zvýšenou dynamiku aktinového cytoskeletu.

1. INTRODUCTION

Cancer is one of the world's leading causes of death. In 2010, it was the main cause of death, responsible for over 8 million deaths worldwide. It has been described as a multistage process, which results in the transformation of a normal cell into a tumour cell. One defining characteristic of cancer is the rapid formation of abnormal cells that grow and invade adjacent parts of the tissue and spread to the distinct target organs, where they seed secondary tumours, termed metastasis. This process is the major cause of death in cancer patients. The most crucial features in the metastatic cancer dissemination are cell invasion and migration, by which the tumour cell overcomes barriers. During their distribution into the neighbouring tissues, cancer cells have to undergo various changes in gene expression, cell-matrix adhesion, remodelling their actin cytoskeleton and other dramatic changes in their signalling pathways. Recent studies have shown that metastatic dissemination of cancer cells is also dependent on changes in tumour microenvironment (Bissell et al., 2002, Hanahan and Wienberg, 2000). During the process of metastases, cancer cells involve several critical steps, which are known as metastatic cascade (Ahmad and Hart, 1997).

1.1 METASTATIC CASCADE

The metastatic cascade includes following steps during which cancer cells detach from the primary solid tumour, penetrate the basement membrane and through the bloodstream or lymphatic system translocate into the target organs, where they seed and proliferate as secondary tumours (Lauffenburger and Horowitz, 1996, Friedl and Wolf, 2003, Friedl, 2004). The process of successful metastasis of malignant cancer cells includes whole series of cellular and molecular changes which are required for overcoming barriers.

It includes:
1. Loss of cell-cell adhesion contact and epitelial-mesenchymal transition
2. Invasion into the surroundings tissues and angiogenesis
3. Intravasation into blood or lymphatic system and transport through the vessels

4. Extravasation from vessels and micrometastasis formation.

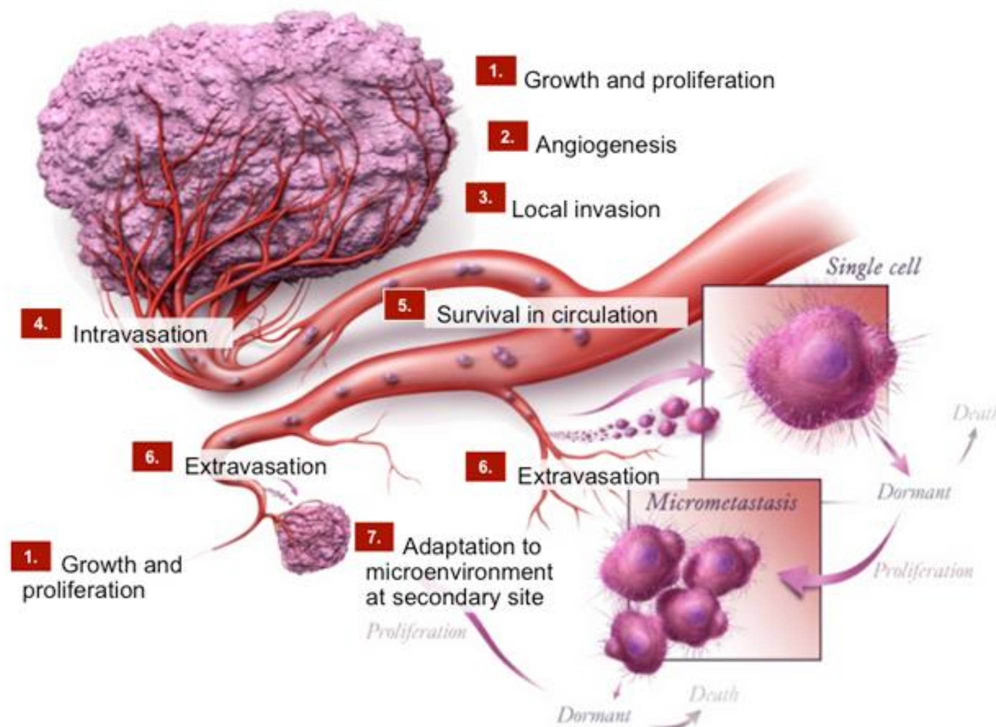

Figure 1. Metastatic cascade. Cells grow and proliferate as benign tumour. After break through the basement membrane the tumour cells invade and enter the bloodstream by process called intravasation. Cancer cells spread and adhere to the capillary walls and extravasate blood system. They adapt to new microenvironment, proliferate and form metastases (adapted from websites by © Agendia 2009-2011).

The epithelial-mesenchymal transition (EMT) is the conversion from multicellular growth and invasion to mesenchymal single cell migration (reviewed in Thiery, 2002). The major mechanism of epithelial-mesenchymal transition is the disruption of the tight epithelial cell-cell adhesion contacts. This process is predominantly caused by downregulation of epithelial protein E-cadherin from the adherent junctions, serving as a suppressor of invasiveness, but also catenins, which are implicated in the regulation of cell-cell adhesion. This low expression of E-cadherins during tumour progression is compensated by upregulation of mesenchymal N-cadherins in a process called “cadherin switch” (Umbas et al., 1994). The cadherin switch results in dramatic changes in the adhesive properties

of metastatic cells and thus enable them to detach from the primary tumour (Hulit et al., 2007, Nieman et al., 1999). During EMT, the cells usually lose their cell polarity and acquire typical spindle-shaped morphology. Importantly, metastazing cancer cells usually produce high amounts of various proteases, which allow them to degrade and thus invade surroundings extracellular matrix (Stetler-Stevenson et al., 1993). Spreading of tumour cells within the tissue activate growth of new vessels, mainly from existing ones by a process called angiogenesis (Holash et al., 1999). Without vascularization, the tumour nodules cannot grow, as they need constant supply of oxygen and nutrients (Folkman et al., 1972).

When tumour cells migrate, they overcome the basement membrane; enter the blood vessels or lymphatic system through the gaps in the endothelial wall, by a process called intravasation (Carr et al., 1980, Wyckoff e al., 2004). In the bloodstream, circulating cancer cells may interact with platelets and leukocytes, to adhere to the endothelium and extravasate from the vessels. These interactions with blood cells are very important for successful extravasation, which is also facilitated by integrin, ezrin and selectin proteins (Wang et al., 2004, Felding-Habermann et al., 2001, Khanna et al., 2004). Spreading of the cancer cells to the specific tissues, their colonization and establishment of the new micrometastasis and secondary tumours is strongly dependent on the tumour cells and microenvironment in the metastatic target organs (Brábek et al., 2010).

1.2 MECHANISMS OF SINGLE CELL TUMOUR INVASION IN 3D ENVIRONMENT

Tumor cell invasion and migration is a process that involves specific patterns and mechanisms. The single cancer cells can employ two different modes of migration in 3-dimensional environment. The first type is a fibroblast-like strategy or mesenchymal migration, for which there is a typical protease-mediated degradation of the extracellular matrix (Sahai and Marshall, 2003). This form of motility is associated with elongated cell morphology, Rac-dependent F-actin protrusions and integrin mediated adhesion (Ridley et al., 1992). The second type is a leukocyte-like strategy or amoeboid migration, which is dependent on the Rho/ROCK signalling pathway. Migrating tumour cells have a typical rounded

shape and they do not require pericellular proteolysis for degradation of ECM (Wolf et al., 2003). Both of these modes of motility use different molecular mechanisms.

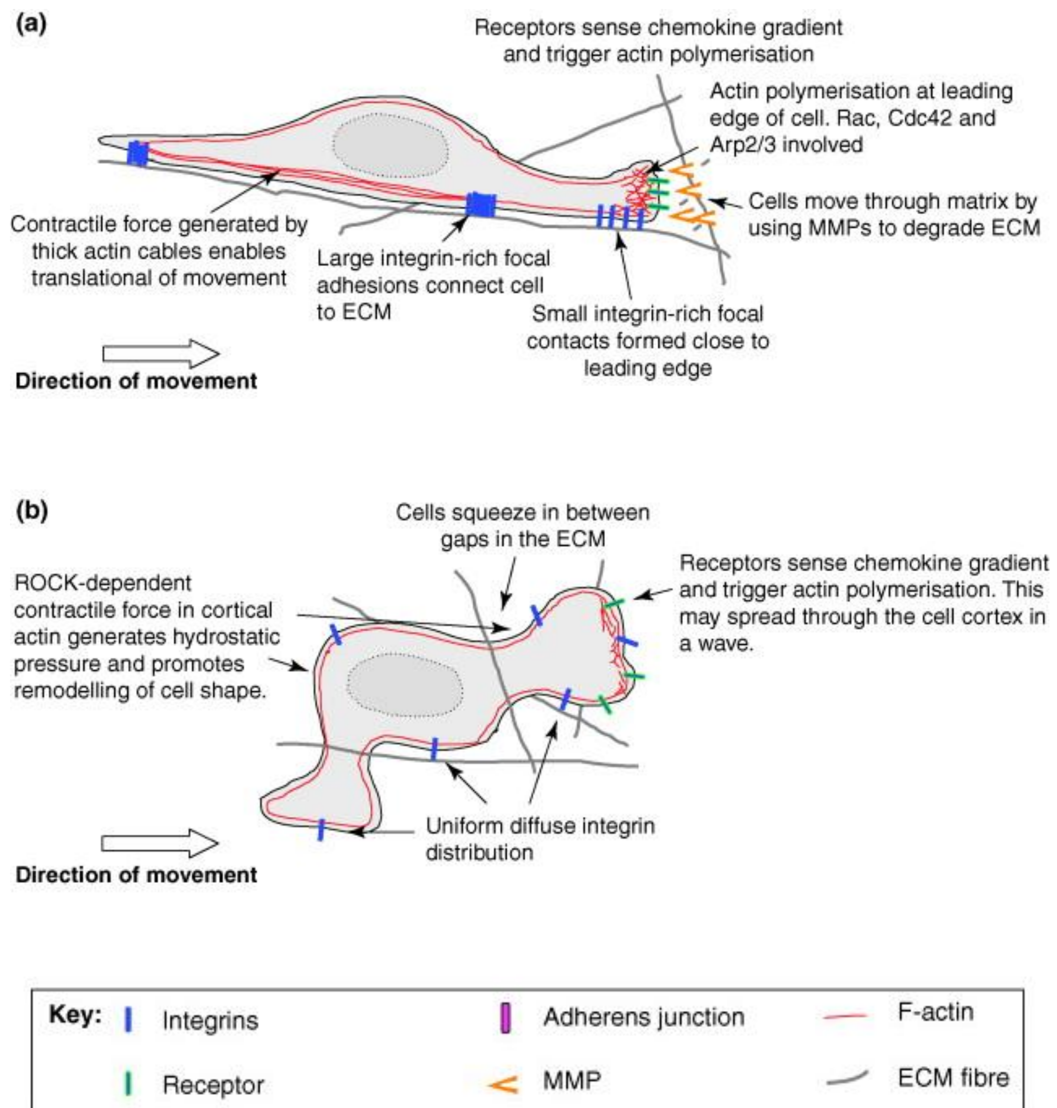

Figure 2. Mechanisms of cancer cell motility. **(a)** Mesenchymal motility is characterized by elongated morphology, and is dependent on MMPs. **(b)** Amoeboid motility is characterized by an amorphous cell shape and rapid changes in direction. (adapted from Sahai E. (2005). Mechanisms of cancer cell invasion. Curr Opin Genet Dev. 15,87-96. Review)

1.3 MESENCHYMAL INVASION

Single cells, using mesenchymal mode of invasion exhibit fibroblast-like morphology, are commonly observed as fibroblasts, keratinocytes, endothelial cell or various types of tumour cells (reviewed in Grinnell, 1994). This specific elongated shape of cell is dependent on β1 and β3-integrin mediated adhesion. As adhesive interactions between the cell and ECM are present on the both cell edges, emerging contractile actin stress fibres, in 2D or cortical cytoskeleton in 3D, generate high traction forces on both of the cell poles (Tamaritz et al., 2002, Ballestrem et al., 2001). One of the most important feature for mesenchymal invasion is upregulated production of activated matrix metalloproteases and other secreted proteases, which are essential for proteolytical cleavage of the extracellular matrix. This degradation and remodelling of ECM enables migration of cells through interstitial tissue barriers (Stetler-Stevenson et al., 1993). Friedl and colleagues have described proteolytic migration of mesenchymal cell in five steps. At the beginning, chemokine or adhesion receptor-mediated signalling results in the formation of pseudopods, functionally alternative to lamellipodia, which are observed in cell migrating in 2D (Wang et al., 2002). After that, integrins interact with laminins in substrate, and form focal adhesion complexes. Increased cell traction force generation is transmitted towards the ECM, which becomes pulled and realigned. The leading edge of cells has a tip-like shape and usually becomes anchored to the ECM. Local proteolysis, together with focalized filamenous actin, clustered integrins and surface proteases. After actomyosin contraction, mediated by myosin II, the rear end moves forward and a track of remodelled ECM becomes apparent. The process of single mesenchymal cancer cell migration is driven by the small Rho-family GTPases Rac1 and Cdc42, which generate formation of actin rich filopodia and lamellipodia at the leading edges of migrating cells (Ridley et al., 1992, Nobes et al., 1995). The velocity of migration by mesenchymal cells is relatively low, approximately 0,1-1 µm/min. (Friedl et al., 1998).

In addtion to proteolytic degradation of the ECM, there are also actin rich adhesion structures, termed as invadopodia and podosomes, which are required for tumour invasion and metastasis. Invadopodia and podosomes are

proteolytically active structures, which contain actin regulatory proteins, such as cortactin, cofillin and talin, and adhesion molecules, such integrins, vinculin and paxillin and proteases (Baldassarre et al., 2003).

Their formation is mediated by small Rho GTPase protein Cdc42-WASP (Wiskott-Aldrich Syndrome protein) /N-WASP (neural WASP)-Arp2/3 complex signalling pathway. WASP and N-WASP proteins belong into the WASP family, involved in actin polymerization and thus cell protrusions (reviewed in Miki et al., 2003, Millard et al., 2004).

1.4 MATRIX METALLOPROTEASES

The mesenchymal invasion is very tightly associated with the pericellular degradation of the extracellular matrix. This process is mediated by the production of matrix metalloproteases (MMP), serine proteases and catepsins (Wolf et al., 2003). The cleavage of components of the ECM and thus its remodelling is one of the first steps in tumour invasion and metastasis. More than 20 proteolytic enzymes have been described, which are able to cleave specific components of the extracellular matrix. MMPs are produced as latent proenzymes. After cleavage of the MMPs prodomain by activating proteases and its dissociation from catalytic domain, metalloprotease becomes activated (Visse et al., 2003).

The structure of matrix metalloproteases contains some accessory domains, which are responsible for the recognition of specific substrate or cellular localization, propeptide domain, catalytic domain, zinc-binding motif and mostly also hemopexin-like domain. Their N-terminal prodomain consists from 80 amino acids and it is responsible for the blocking of catalytic activity via organization of a conserved cysteine residue, within a PRCGxPD motif “the cysteine switch” to the catalytic zinc (Visse et al., 2003, Tallant et al., 2010).

The catalytic domain of MMPs has a globular shape containing 160–170 amino acids with highly conserved HExxHxxGxxH zinc binding motif. This motif it seems to be responsible for chelating the catalytically critical zinc ion at the enzyme active site (Tallant et al., 2010). Catalytic domain also contains a region for the binding of endogenous tissue inhibitors of metalloproteinases (TIMPs) (Nagase et al., 2006). Moreover, catalytic domain of gelatinases such as MMP-9

and MMP-2 is enriched by three fibronectin repeats that are important for binding to gelatin, collagens, and laminin (Opdenakker et al., 2001).

C-terminus of most MMPs may contain hemopexin domain, which is connected via flexible linker to the catalytic domain (Bertini et al., 2009). It has been described that hemopexin domain mediates recognition of the specific binding substrates as well as interaction with tissue inhibitors TIMPs (Roeb et al., 2002, Monaco et al., 2007). Moreover interactions between PEX domains and integrins in mammary epithelial cells facilitate MMP activation, and localize soluble MMPs to sites of proteolysis (Piccard et al., 2007).

Membrane-bound metalloproteases (MT-MMPs) are cell membrane-anchored proteases with a transmembrane domain, moreover some of them share a glycosyl-phosphatidyl inositol anchor (reviewed in Sohail et al., 2008).

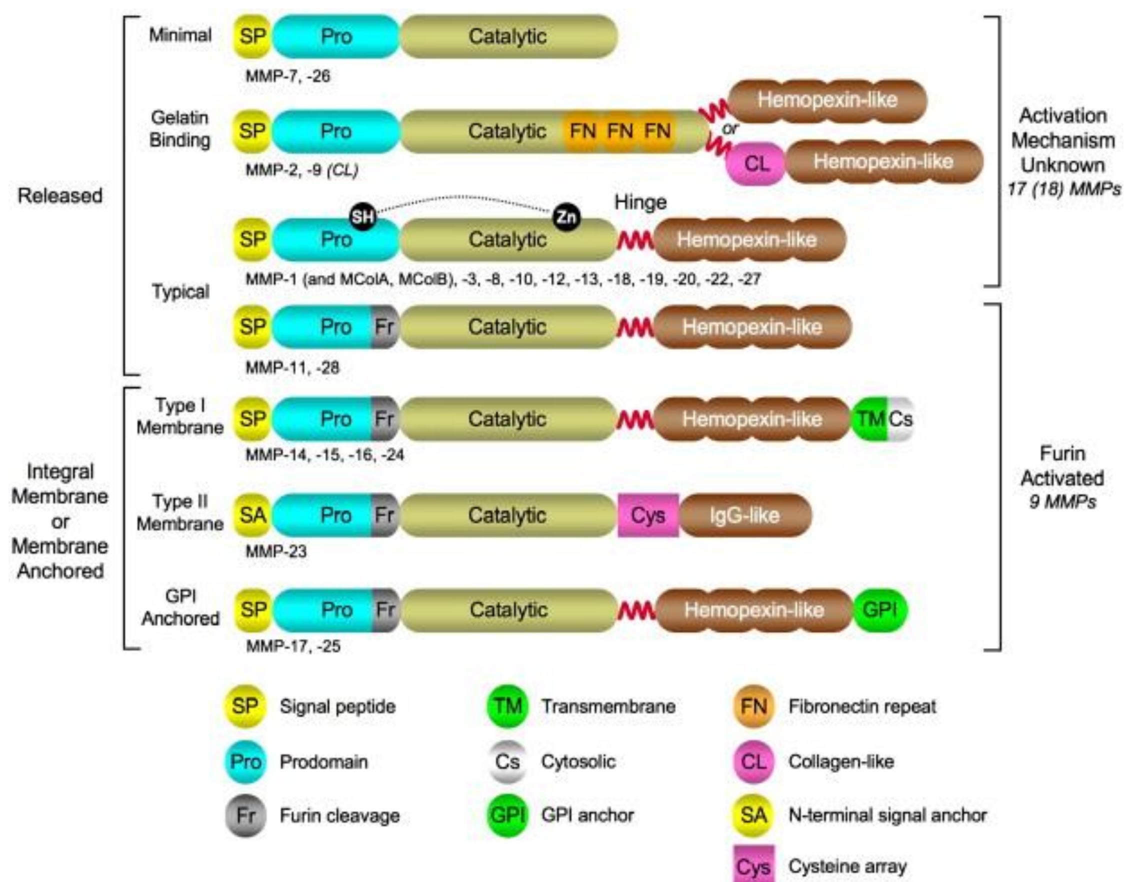

Figure 3. Structures of mammalian MMPs. (adapted from Hyun-Jeong Ra and William C. Parks (2007). Control of Matrix Metalloproteses catalytic Activity. Matrix Biol, 26, 587–596).

Metalloproteases can be regulated by inhibitors, such as α2-macroblobulin and thrombospondin, which controls clearance of extracellular MMP-2, or by more specific tissue inhibitors (TIMPs). Four TIMP inhibitors have been identified, TIMP1-4. All TIMP inhibitors share twelve cysteine residues that form six disulphide bonds, and created conformations are essential for the inhibition of MMPs. The C-terminus of all TIMPs contains various regions, which are necessary for binding specificity to MMPs. It has been reported that TIMPs inhibit MMPs by creating a strong noncovalent complex in stechiometry 1:1. Nevertheless, various MMPs can be inhibited by different TIMPs (Bigg et al., 1997, Douglas et al., 1997).

Many studies have pointed at strong correlation between overexpression of metalloproteases in tumour cells and their malignant progression. For example, it has been observed that MMP-9 and MMP-2 overexpression, and their activity, strongly correlates with the process of metastasis and poor prognosis (Lubbe et al., 2006). MMP-9 has been found as a regulator of the activity of another serine protease: urokinase-type plasminogen activator uPA. Its degradation of uPA inhibitor nexin-1 promotes invasion of tumour cells (Xu et al., 2010).

Many studies have also reported that MT1-MMP is the key regulator of invasion and metastasis via activation of proMMP-2 and thus direct degradation of collagen type I, II and III (Poincloux et al., 2009). Furthermore, MT1-MMP plays the important role in growth of tumour cells during their invasion (Nannuru et al., 2010, Sabeh et al., 2009).

For process of tumour progression, the balance is critical between inhibitors of MMPs, such as TIMPs and activated MMPs. It has been shown that decreased expression of TIMPs leads to increased expression levels of activated MMPs, and thus to increased capability of cancer cells. There is also evidence that overexpression of TIMPs can also act in tumour suppression. The ability of TIMPs to inhibit tumour invasion and metastasis, as well as early and late stages of tumorigenesis, was documented (Watanebe et al., 1996, Stetler-Stevenson et al., 1993). Proteases enriched in structures are called invadopodia. Coordinated adhesion to the substrate and proteolytic interaction with the extracellular matrix can be mediated either by membrane-bound proteases, which bind to the β1 or β1-

integrins, or by interaction of soluble metallopoteases to the cell receptors (Deryugyna et al., 1998).

1.5 AMOEBOID TYPE OF INVASION

The cells use the amoeboid mode of migration share a typical “rounded” morphological feature, characterized as “amoeboid”. They are translocated with fastly repeated cycles of expansion and contraction, and in comparison to the mesenchymal motility, the velocity of amoeboid movement is also higher, about 2-30 μm/min. (Friedl et al., 1998). It was shown that different amoeboid cell types exhibit various mechanisms which lead to two typical amoeboid phenotypes described as either contraction-based blebbing, or the second type as the entire actin polymerization-driven migration (Lammermann et al, 2008).

A few years ago, it was surprisingly discovered that amoeboid tumour cell invasion is independent on proteolytic activity, which is required for matrix degradation. This finding was very important for the elucidation of metastatic cancers, and the reason for poor results obtained when using metalloproteases inhibitor treatment in clinical trials (Overall et al., 2006). Following a very detailed investigation, the new type of invasion was established, called amoeboid.

It was shown that amoeboid invasion is enabled by forced generation of polarized actomyosin cytoskeleton carried out by upregulation of RhoA/ROCK signalling pathway (Watanabe et al., 2005, Rosel et al., 2008). Furthermore, it was also suggested that amoeboid motility, promoted by ROCK upregulation, is very tightly correlated with the increased metastatic potential of cancer cells (Wyckoff et al., 2000, Rosel et al., 2008).

During amoeboid movement, the cortical actin dynamics is controlled by small GTPase protein RhoA, and its effector Rho-associated serine threonine protein kinase ROCK. Rho/ROCK signalling promotes the formation of a dense contracting actomyosin mesh at the invading cell edge (Wyckoff et al., 2006). Notably, in amoeboid migrating tumour cells there were neither stress fibres nor visible focal contacts (Friedl et al., 1998, Wolf et al., 2003).

ROCK can regulate actomyosin activity by direct phosphorylation of MLC and it is localized at the leading edge of the migrating cell, immediately behind

actin-rich protrusions. Moreover, the ROCK-dependent phosphorylation of MLC is crucial for its correct organization and force generation that enables cell movement within ECM without proteases. ROCK also regulates the localization of MLC to the cell cortex (Wyckoff et al., 2006). In the Rho/ROCK signalling pathway, the one major substrate for ROCK is the myosin binding subunit of myosin phosphatase, MLCP, which dephosphorylates myosin II light chain MLC2. Phosphorylation of regulatory subunit of MLCP at Thr-853 and Thr-696 by ROCK leads to its inactivation (Kimura et al., 1996). ROCK phosphorylates and activates LIMK that leads to phosphorylation and thus inactivation of cofilin that was described as actin-depolymerization factor (Maekava et al., 1999, Amano et al., 2010). MLC2 can also be phosphorylated and activated by myosin light chain kinase MLCK (Amano et al., 1996). RhoA/ROCK signalling cascade is implicated in the stiffness generation and maintenance of roundish cell morphology of the cells (Worthylake et al., 2001, Smith et al., 2003).

As it was mentioned above, the extracellular proteases or calpain are not required for amoeboid migration, due to the enhanced contractile force at the leading edges of cells, which promotes them to squeeze through the gaps and spaces within the extracellular matrix (Sahai and Marshall, 2003). It has been observed, that during 3D migration the cells adapt their cytoplasm and nucleus to minimize resistance towards tissue barriers in both mesenchymal and amoeboid migration (Wolf et al., 2003). Moreover, Provenzano and his colleagues have shown that migrating tumour cells that use Rho/ROCK mediated forces, generated by cellular myosin-based contractility are able to actively deform and reorganize the three-dimensional collagen matrice (Provenzano et al., 2008). It was observed that migrating cells, using the amoeboid type of invasion, form only "short-lived" and relatively weak integrin-free interactions without tightly adhering to the adjacent environment (Lammermann et al., 2008, Friedl et al., 1998, Werr et al., 1998).

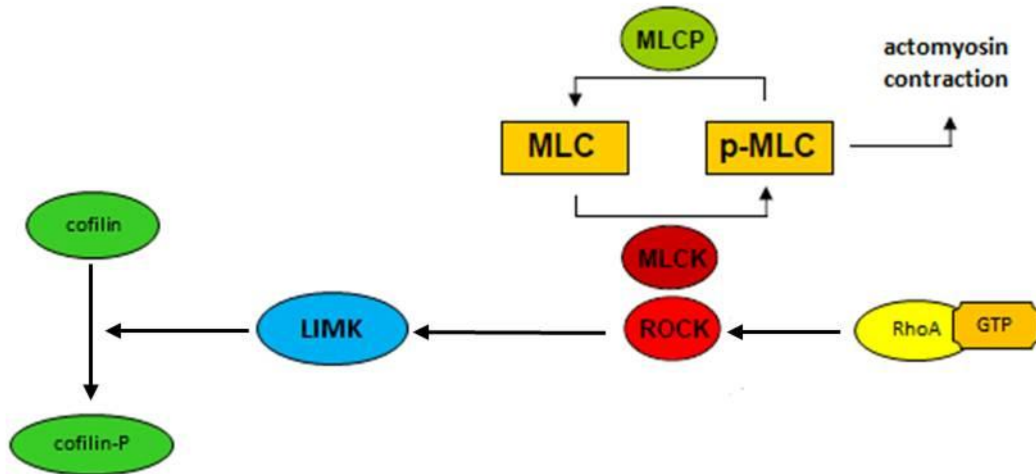

Figure 4. Rho/ROCK mediated actomyosin contraction involved in amoeboid mode of invasion. RhoA signals through ROCK to increase MLC phosphorylation that generates actomyosin contraction. Phosphorylation of LIMK leads to phosphorylation and inactivation of cofilin.

The amoeboid tumour cells also use blebbing movement as another migratory strategy. The blebbing migration is driven by a hydrostatic pressure gradient and it has been considered as an alternative mechanism to the lamellipodia-driven movement in 3D (Charras et al., 2005). The blebs are preferently developed at the leading edges of the moving cells, and their formation in cancer cells is also associated with enhanced migration and invasiveness (reviewed in Fackler et al. 2008, Charras et al., 2008). The blebbing migration requires myosin activation and its contraction, which is generated by small GTPase, RhoA, and its effector, Rho-kinase ROCK or myosin light chain kinase MLCK (Yanai et al., 1996, Coleman et al., 2001). This signalling pathway leads to contraction of the cortical actin meshwork at the sides and rear of the cells and thus generates hydrostatic pressure (Wyckoff et al., 2006).

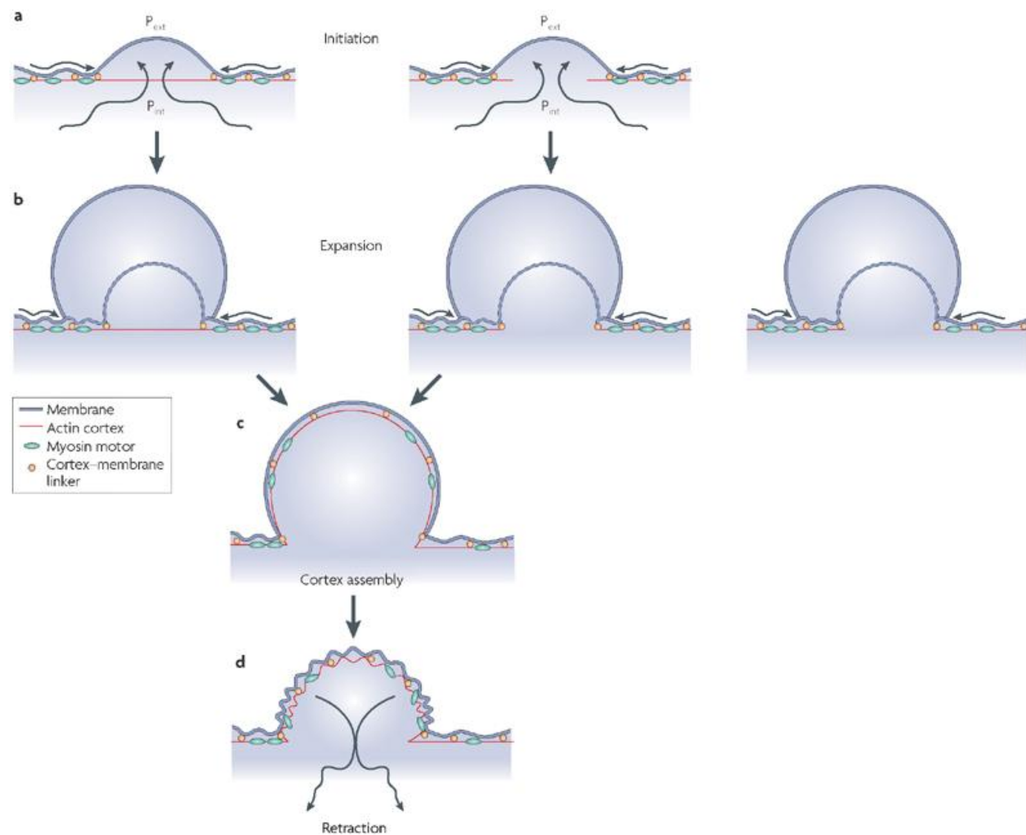

Figure 5. The three phases of bleb cycle: bleb initiation (nucleation), expansion and retraction. a) Bleb initiation can result from a local detachment a local detachment of the cortex from the membrane (left model) or from a local rupture of the cortex (right model). **b)** Hydrostatic pressure in the cytoplasm (Pint) then drives membrane expansion by propelling cytoplasmatic fluid through the remaining cortex (left model) or through the cortex hole)right model). Concomitantly, the membrane can detach further from the cortex, increasing the diameter of the bleb base (dashed line). **c)** As bleb expansion slows down down, a new actin cortex reforms under the bleb membrane. **d)** Recruitment of myosin to the new cortex is followed by the cbleb retraction (Pext) extracellular hydrostatic pressure (adapted from Charras and Paluch (2008). Blebs lead the way: how to migrate without lamellipodia. Molecular Cell Biology. 9, 730-736).

1.6 MESENCHYMAL-AMOEBOID TRANSITION

Many reports have shown that migrating tumour cells can change their mode of migration. This conversion can be influence by many conditions. It has been

named mesenchymal-amoeboid (MAT) or amoeboid-mesenchymal transition (AMT). It has been reported, that one of the reason of MAT could be the blockage of proteases in tumour cells. Wolf and colleagues found out that this transition, from proteolytic mesenchymal to nonproteolytic amoeboid cell migration, represents a putative escape mechanism in tumour cell dissemination after abrogation of pericellular proteolysis. It has been shown, that mesenchymally migrating, highly invasive fibrosarcoma HT-1080, as well as MDA-MB-231 mammary carcinoma cells, that exhibit spindle-shaped morphology, have converted to less polarized and more spherical "amoeboid" morphology in the presence of proteases inhibitors (Wolf et al., 2003). This induced proteases-independent "amoeboid" migration is caused by cellular and molecular adaptation of tumour cells on environmental conditions and may represent an escape mechanism upon protease inhibitor-based therapy in aggressive tumour cells (Zucker et al., 2000, Kruger et al., 2001). Another known mechanism that leads to MAT is the activation of the Rho/ROCK signalling pathway.

As mentioned above, Rho/ROCK signalling is implicated in actomyosin contractility, as well as in the maintenance of a rounded shape of migrating cells. Sahai and colleagues have observed that constitutive activation of ROCK kinase results in transition from mesenchymal to amoeboid migration in HT1080 cells (Sahai and Marshall, 2003). Conversely, silencing of Rho/ROCK pathway in amoeboidly migrating A375M2 melanoma cells leads to the mesenchymal phenotype. A very recent study has shown that silencing of NG2/MSCP transmembrane proteoglycan results in transition from amoeboid to mesenchymal migration in both melanoma A375M2 and rat sarcoma A3 cells. This inhibition of NG2 led not only to conversion in phenotype but also to increase in proteolytic degradation of components of the extracellular matrix (Paňková et al., 2012).

1.7 NG2 PROTEOGLYCAN

NG2/CSPG 4 chondroitin sulphate proteoglycan 4 is 250 kDa, membrane-bound, cell surface protein. It was first identified in rat as a Neuron – glia 2 progenitor marker (Stallcup et al, 1981, Nishiyama et al., 1991). It is also known as High Molecular Weight-Melanoma Associated Antigen (HMW-MAA) or

melanoma chondroitin sulphate proteoglycan (MSCP) (Pluschke et al, 1996). Its homolog was found in mouse as neural antigen 2 (AN2) (Niehaus et al., 1999), as well as in C. elegans as C48E7.6.p and in Drosophila as CG10275. (Celniker et al., 2002). NG2 was found widely expressed by several tissue specific immature progenitor cells during development, including glial precursor cells, skeletal myoblasts, chondroblasts of developing cartilage, and brain capillary endothelial cells (Nishiyama et al., 1991, Grako et al., 1995, Burg et al., 1999, Schlingemann et al., 1990, 1996).

NG2/MSCP as a membrane-spanning protein facilitates the communication between extracellular environment and intracellular compartments of the cell. It belongs to the proteoglycan family, which contains glycosaminoglycan chains that are covalently linked to their core protein. NG2/MSCP, as well as several other proteoglycans, are known for their involvement in various aspects of cells behaviour, including cell/matrix interaction and migration (Rapraeger et al., 1986, Perris et al., 1987), cell proliferation (Yamaguchi and Rouslahti, 1988) and cell adhesion (Yamagata et al., 1989). Interestingly, there is growing evidence that NG2/MSCP proteoglycan has a significant role in tissue invasion, metastasis and tumour progression (Burg et al, 1999, Iida et al., 2001).

Recent work has demonstrated the association of NG2 with the tumour progression and poor prognosis (Cattaruzza et al., 2008, Benassi et al., 2009). Upregulation of NG2 proteoglycan was found in different types of tumours as melanomas (Burg et al., 1998), soft tissue sarcomas (Benassi et al., 2009), glioblastomas (Shoshan et al., 1999) and acute myeloid leukemia (Behm et al., 1996, Mauvieux et al., 1999). Moreover, a recent study also showed that NG2 also promotes wound healing and neovascularization of a tumour mass (Stallcup and Huang, 2008, Schlingemann et al., 1990, Levine 1994). Increased tumour angiogenesis allows metastases formation and tumour growth (Burg et al, 1998, Chekenya et al, 2002). These processes, mediated by NG2 proteoglycan, are very important for tumour invasion and also strongly influence the metastatic potential of cancer cells (Chekenya et al., 2002, Burg et al., 1997, Makagiansar et al., 2007). As the strong correlation between the expression of NG2 proteoglycan and metastases formation, in a soft-tissue sarcoma patient, has been confirmed, NG2 could be used and considered as a predictive marker for metastatic formation (Cataruzza et al., 2008). In addition, Cavanna and colleagues also showed that

NG2 expression is strongly upregulated in highly metastatic sarcoma cells (Cavanna et al., 2007), which use the amoeboid mode of invasion (Rosel et al., 2008). This data points out the fact that NG2 proteoglycan could be the long-term searched, non-integrin adhesion molecule involved in amoeboid mode of invasion (Paňková et al., 2012).

1.8 STRUCTURE OF NG2 PROTEOGLYCAN

NG2, a chondroitin sulphate proteoglycan, is a type 1-transmembrane protein, which is initially synthesized as a 250 kDa polypeptide. NG2 consists of 3 domains: extracellular, transmembrane and cytoplasmatic. The large extensive extracellular domain has 2225 amino acids, transmembrane domain with 26 residues and very short 76 amino acid cytoplasmatic domain. Unfortunately, there are no 3-dimensional structural data for NG2.

The extracellular domain consists of three domains. An ectodomain, which is composed of two globular domains that form a structure described as “dumbbell-shaped” (Tillet et al., 1997), and it is stabilized by intramolecular disulfide bonds (Nishiyama et al., 1991). This ectodomain contains two laminin G-type specific motifs that are important for ligand binding (Stegmuller et al., 2002). In the central domain of the NG2 ectodomain, there is a consensus sequence at ser-999 for chondroitin sulphate attachment, (Stallcup and Dahlin-Huppe, 2001) binding sites for collagen V and collagen VI (Burg et al., 1997, Tillet el al., 1997), and a glycosaminoglycan chain. There is growing evidence that the glycosaminoglycan chain is important for targeting the NG2 molecule to specific molecules within microdomains of the cell membrane (Stallcup and Dahlin-Huppe, 2001).

The globular, juxtamembrane part of the extracellular ectodomain of NG2 contains N-linked oligosaccharides that are involved in the binding of galectin-3 (Fukushi et al., 2004). In this domain, there are also binding sites for β-1 integrins and sites for proteolysis of NG2. This proteolytic cleveage leads to its release from the cell surface (Nishiyama et al., 1995) and its enhanced presence has been observed in some types of injuries (Jones et al., 2002).

The cytoplasmatic domain of the NG2 proteoglycan includes a few motifs and several cytoplasmatic threonine residues, which are essential for the correct

function of NG2. The C-terminus of the cytoplasmatic domain contains a PDZ-binding motif, QYWV, which is involved in the interaction with multi-PDZ scaffold protein MUPP1 (Barrit et al., 2000), GRIP1 (Stegmuller et al., 2003) and syntenin-1 (Chatterjee et al., 2008). In the cytoplasmatic domain of NG2, there is also a proline-rich segment and one serine and seven threonine residues (Nishiyama et al 1991), however only two of them are phosphorylated. The Thr-2256 is phosphorylated by PKCα, and this phosphorylation leads to enhanced motility. The second phosphorylation is catalyzed by ERK at threonine 2314, and is responsible for stimulating cell proliferation (Makagiansar et al., 2004).

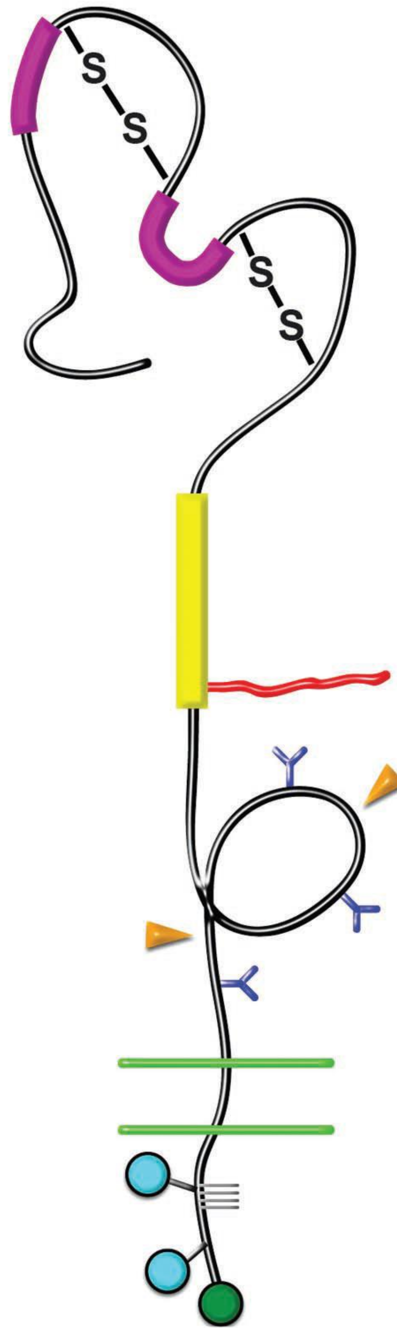

Figure 6. Structure of NG2. **Extracellular domains:** ectodomain 1: violet bars, with laminin G domains and S-S, disulfide bonds**.** Ectodomain 2: yellow bar with collagen binding domain; irregular red line represents chondroitin sulfate chain. Ectodomain 3: Blue Y-shapes, N-linked oligosaccharides; orange arrowheads represent sites of proteolytic cleavage. **Transmembrane Domain:** double green lines. **Cytoplasmic Domain**: blue circles, sites of threonine phosphorylation; green circle, PDZ binding motif; gray grid lines, proline-rich segment. (adapted from Willam B. Stallcup and Feng-Ju Huang. (2008). A role

for the NG2 proteoglycan in glioma progression. Cell Adhesion and Migration, 2, 192-201).

1.9 NG2 PROTEOGLYCAN AND ITS FUCTION IN AXON REGENERATION

Several studies have indicated the potential connection of NG2 proteoglycan with the Rho/ROCK signalling pathway. The first clue came from the axon regeneration study. The researchers have pointed on inhibitory effect of the CSPG4 proteoglycans on neurite outgrowth in vitro and axonal growth in vivo (Dou and Levine, 1997, Gates et al., 1996, McKeon et al., 1991). Even if the mechanism is still not entirely known, recent studies have discussed the potential involvement of the Rho/ROCK signalling pathway (Fournier et al., 2003, Borisoff et al., 2003, Monnier et al., 2003).

It has been reported that activation of the RhoA/ROCK signalling pathway, in neurons, has an inhibitory effect on axon growth that results in a neurone cone collapse and its retraction. These data has been supported by experiments in which treatment with pharmacological inhibitors of both RhoA and ROCK lead to neurite growth of retinal axons and outgrowth of dorsal root ganglion cells in vitro (Monnier et al., 2003, Fournier et al., 2003). In addition, in vivo blocking of RhoA by C3 exoenzyme, as well as using of nonsteroidal anti-inflammatory drugs against RhoA, caused axon regeneration (Fournier et al., 2003). To confirm the inhibitory effect on neurite outgrowth, experiments were performed on CSPG-rich substrates. The axonal regeneration was observed in the presence of ROCK inhibitors (Borisoff et al., 2003, Monnier et al., 2003), as well as after the treatment with NG2 antibody (Dou and Levine, 1994).

A very recent study also reported the possible interconnection between NG2 proteoglycan and Rho/ROCK signalling pathway in tumour cells. Authors have shown that NG2 mediated activation of Rho is tightly associated with amoeboid invasiveness of tumour cells (Paňková et al., 2012).

1.10 NG2 AND ITS POSSIBLE INTERACTION WITH Rho/ROCK SIGNALLING PATHWAY

There is growing evidence that NG2 mediates remodelling of the actin cytoskeleton, which leads to migration and invasion of tumour cells via the activation of Rho family GTPases (Lin et al., 1996, Eisenman et al., 1999, Majumdar et al., 2003). Nevertheless the mechanism is still not entirely clear. A recent study has reported that downregulation of NG2 leads to inactivation of Rho GTPase which is associated with reduced invasiveness of tumour cells (Paňková et al., 2012).

Many recent studies have indicated that the interconnection of NG2 proteoglycan with Rho/ROCK signalling pathway is indirect, and it is mediated by a scaffold protein. One of the most presumptive candidates is MUPP1, which has already been identified as a binding partner for the cytoplasmatic domain of NG2. MUPP1 is a scaffold protein also implicated in many other signalling pathways. It is multi PDZ domain-containing scaffold protein, which directly binds to the PDZ binding motif of NG2 proteoglycan (Barrit et al., 2000). The paralogue of MUPP1, Patj, has been identified as a scaffold protein in the Amot:Patj/MUPP1:Syx ternary complex activating the RhoA signalling pathway, which leads to the migration of endothelial cells. Importantly, this complex controls the activity and localization of RhoA at the leading edges of migrating cells (Erngvist et al., 2009).

Angiomotin (Amot) is the membrane-associated protein with a C-terminal PDZ binding motif. Syx has been recognized as a RhoA GTPase exchange factor (Rho-GEF), which is responsible for activating small GTPases (Ridley et al., 2001). Because Syx contains a RhoGEF domain, a plextrin homology domain and only a PDZ-binding motif at its C-terminus, its interaction with angiomotin is mediated via the scaffold protein Patj/MUPP1 (Erngvist et al., 2009). There is thus a possibility that NG2 controls local GTPase activity through NG2/MUPP1/SYX/RhoA complex.

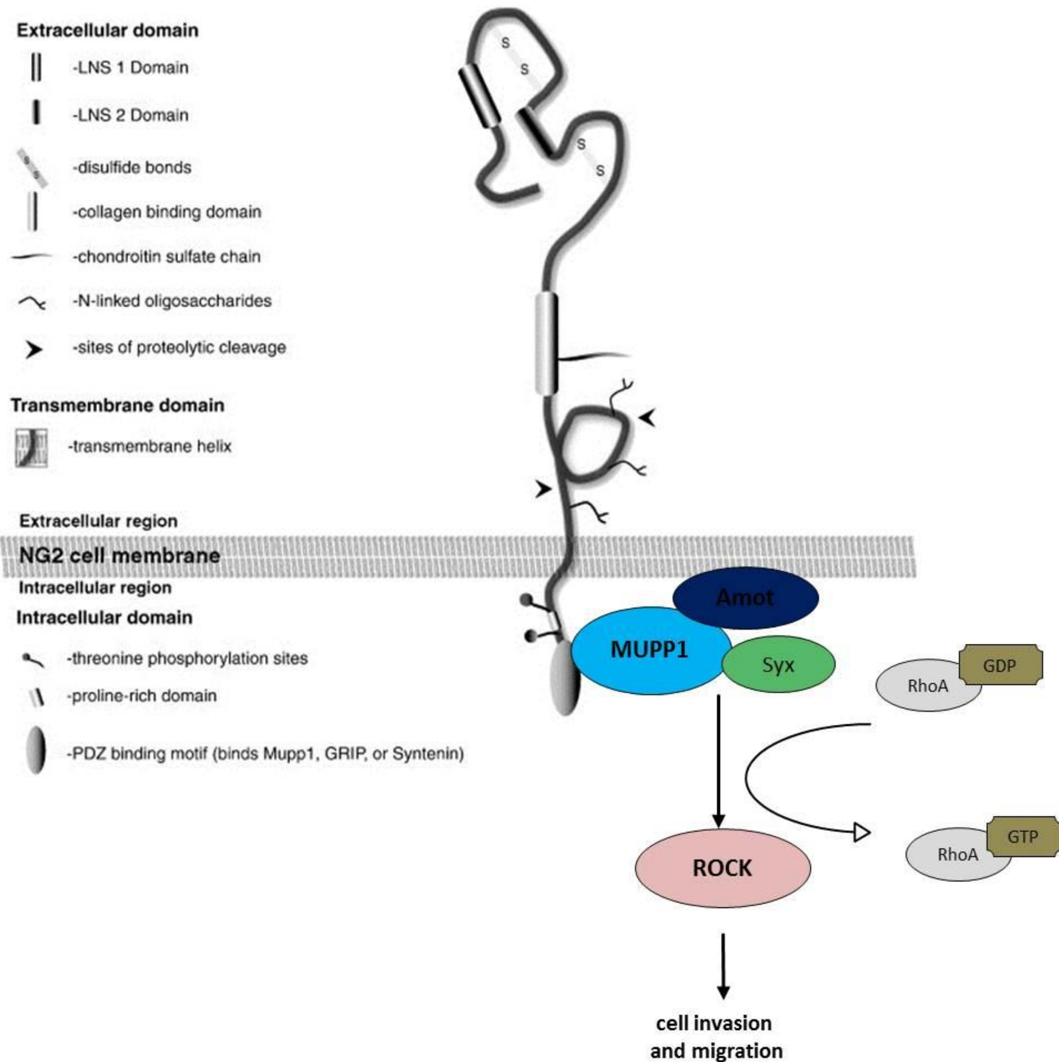

Figure 7. Possible mechanism of connection between NG2 proteoglycan and Rho/ROCK signalling pathway. (adapted from Trotter et al.,(2010) NG2 cells: Properties, progeny and origin. Brain Research Rev. 63,72-82).

1.11 THE FUNCTION OF NG2/MSCP IN TUMOUR CELLS

The possible mechanism by which NG2 influences motility is by the activation of Rac or Cdc42 GTPases, which directly activate Pak-1, a signal molecule responsible for the regulation of cell morphology and cell motility (Sells et al., 1999). Pak-1 also regulates myosin light chain phosphorylation, although NG2 can also promote cell migration and cell spreading.

NG2 is often regarded as a co-receptor mediating ligand-dependant activation. Previous studies have demonstrated that NG2 interaction with hyaluronic acid, fibronectin or CD44 negatively influences adhesion of melanoma cells (Burg et al., 1998). NG2 ability to activate ß1-integrins has also been described (Iida et al., 1995, Eisenmann et la., 1999). Chondroitin sulphate glycosaminoglycan chain is required for this specific activation, which mediates the linkage between NG2 and ß1-integrins, and it is a component of the NG2 extracellular domain (Iida et al., 1998). NG2-mediated ß1-integrin activation promotes cell adhesion, cytoskeletal reorganization and spreading of melanoma cells (Iida et al., 1995, Eisenmann et al., 1999, Fang et al., 1999). NG2-mediated cell spreading is promoted via activated Rho-family GTPase Cdc42 and its effector Act-1 (activated Cdc-42-associated kinase), which phosphorylates p130cas and thus recruits ß1-integrin (Eisenmann et al., 1999). Furthermore, NG2-dependent ß1-integrin activation also has an effect on cell survival through PI3K/AKT pathway (Chekenya et al., 2008).

As it was mentioned above, NG2/MSCP is implicated in migration and proliferation of cancer cells (Burg et al., 1996, Tillet et al., 1997). NG2-driven migration and proliferation can be regulated by two different phosphorylations of NG2 cytoplasmatic domain at two different threonines. It seems that both phosphorylation and dephosphorylation of NG2 could be very important not only for NG2 interaction with others molecules but also for surface localization of NG2. As it has been previously reported, the non-phosphorylated form of NG2 is localized at the small plasma membrane protrusions, which are widely presented on most of the cell surface and can serve as a scaffold for various molecules that mediate communications between cells and the extracellular matrix (Garrgues et al., 1986).

Although, recent studies have shown that NG2/MSCP can be phosphorylated by PKCα or ERK at its two cytoplasmatic threonines, and both phosphorylated stages mediate two different processes. First, PKCα phosphorylates NG2 at Thr-2256 at its cytoplasmatic domain, resulting in enhanced cell motility. After this phosphorylation, NG2 is translocated to the lamellipodia at the cell periphery prominently to the leading edges of the cells. Even if the mechanism of NG2 relocation is not entirely clear, its redistribution is accompanied by a noticeable increase in cell motility (Makagiansar et al., 2004). This phosphorylation could also modify the interaction of NG2 with the cytoplasmatic binding partners or extracellular ligands.

The second phosphorylation is mediated by ERK at Thr-2314 of the cytoplasmatic domain of NG2/MSCP and leads to NG2-dependent proliferation. In addition, this phosphorylation localizes NG2 to the apical microprotrusions and it seems to be also responsible for their formation (Makagiansar et al., 2007). Makagiansar and colleagues showed that signals generated via Thr-2256 phosphorylation by PKCα are dominant over the ERK-mediated phosphorylation at Thr-2314. Conversely, phosphorylation by PKCα has an inhibitory effect on cell proliferation and ERK mediated phosphorylation at Thr-2314 blocks the motility of cells. Although, the mechanism by which both phosphorylations are regulated is still not clear. One possible mechanism is the conformational change of NG2 after its phosphorylation by PKCα.

Previous studies have reported that both phosphorylations of NG2 are dependent on clustering with β_1 integrins. They have discussed two possible mechanisms which control both phosphorylations. It was suggested that phosphorylated variants of NG2 could be differentially influenced by interacting with the different β-integrin heterodimers; or the second possibility that NG2 phosphorylation pattern determines various localization of NG2-integrin complexes, and thus it leads to integrin signalling in the different microdomains of the cells (Makagiansar et al., 2007).

NG2 was described as a potential regulator of membrane microdomains in melanoma cells, where it contributes to the cellular adhesion (Garrigues et al., 1986). NG2 is colocalized with ezrin, PKCα and $\alpha_3\beta_1$ integrin in these NG2 rich microspikes and membrane protrusions (Fukushi et al., 2004, Makagiansar et al., 2004, Ng et al., 2001). Unfotunately, there is no evidence to support the indication

that there is a direct interaction between ezrin and NG2. This could potentially be a co-mechanism of linkage to the actin cytoskeleton (Lin et al., 1996).

1.12 NG2/MSCP AND CELL INVASION

Recently, there is growing evidence which suggests NG2/MSCP as a potential therapeutic target. Previous work has highlighted the implication of NG2 in aggressive phenotypes in human melanoma, gliomablastomas and myeloid leukemias (Chekenya and Pilkington, 2002, Shoshan et al., 1999). As NG2/MSCP serves as a signal transduction molecule, many publications have discussed its interaction with collagen type VI as a possible mechanism involved in invasion and metastasis in cancer cells (Stallcup et al., 1990, Burg et al., 1996, Tillet et al., 1997). It is known that collagen VI is an essential component of the extracellular matrix, where it forms a flexible, microfibrillar network in various connective tissues (Chu et al., 1987). It specifically binds to the binding site found in the central part of the extracellular domain of NG2 (Burg et al., 1997, Tillet et al., 1997). Previous studies have shown that the interaction between collagen VI and NG2 proteoglycan, besides tumour progression, also controls cytoskeletal rearrangement, cell spreading, formation of lamellipodia (Tillet et al., 2002), all accompanied by enhanced cell motility (Burg et al., 1997). NG2 binding to the collagen VI preferentially activates ERK-PKCα-MAPK cascade, which is associated with modulation of cytoskeletal dynamics.

Studies with animal models have shown that NG2 positive tumours grew more rapidly. Furthermore, NG2 overexpression induced highly aggressive tumours accompanied by increased angiogenesis and invasive tumour properties. Conversely, disruption of NG2 function significantly reduced tumour growth and angiogenesis, as well as proliferation (Wang et al., 2010).

It was reported that NG2 interaction with one of its binding partner galectin-3 mediated malignant properties (Fukushi et al., 2004). Galectin-3 belongs to the family of mammalian lectins. Lectins usually contain conserved carbohydrate recognition domains (CRDs), which are responsible for oligosaccharide-dependent binding to the ligand (Liu and Rabinovich, 2005). This CRD domain of galectin-3 also serves as a mediator of binding to the N-linked oligosaccharides

within the extracellular domain of NG2. Wen and colleagues showed that disruption of the NG2/galectin-3 interaction led to a decrease in aggressive properties of tumour cells (Wen et al., 2006).

Membrane-type 3 matrix metalloprotease (MT3-MMP) is another extracellular binding ligand for NG2. The presence of the chondroitin sulphate chain of NG2 is necessary for activation and direct binding to MT3-MMP. This complex is crucial for local invasion and degrading on type I collagen in melanoma cells. (Iida et al., 2007)

2. DISCUSSION

My postgraduate study upon supervision of associate Professor Jan Brábek in the Laboratory of invasion of sarcoma cells was focused on characterization of mechanisms of invasion in tumour cells.

1. The first part of my study was concentrated on the determination of molecular mechanism of amoeboid invasion in rat sarcoma cells. I have also analysed the role of cytoskeleton in invasiveness of the primary breast tumour cells.
2. The second part of my study was to determine the role of NG2/MSCP in amoeboid type of invasion and its implication in Rho/ROCK signalling pathway.

Up-Regulation of Rho/ROCK Signalling in Sarcoma Cells Drives Invasion and Increased Generation of Protrusive Forces
(the 1th. publication)

To analyse the mechanisms of sarcoma cells invasion, we have used the rat sarcoma model of metastasis, which was developed by Pavel Veselý in inbred Lewis rats. The model includes four populations of sarcoma cell lines with different capacities to induce metastases in animals. Cell populations have been developed by isolation from neoplastic rat embryo fibroblast cells and then

spontaneously transformed in vitro. To compare differences, we have analysed the parental non-metastatic fibroblast-like LW13K2 (K2) and highly metastatic A337/377RP (A3) cells, derived from K2 cell line. Both cell populations are closely, genetically related, that it offers specific advantage for studying in vitro metastatic transformation (Cavanna et al., 2007).

Because sarcoma cells are from mesenchymal origin and their mesenchymal invasion is dependent on degradation of extracellular matrix by the production of proteases, we have analysed their gelatinase activity. We surprisingly have found out that highly metastatic sarcoma A3 cells secreted only very low levels of the inactive pro-form of MMP-2 and no active form of MMP-2 protease as well as we have not observed any gelatine degradation of A3 cell seeded on FITC-gelatine. Conversely non-metastatic K2 cells exhibited low level of gelatinase activity that was sensitive for proteases inhibitors. These findings have suggested us to hypothesize that A3 mesenchymal sarcoma cells can use alternative, protease-independent mode of invasion.

Subsequent KINEX protein microarray analysis between K2 and A3 sarcoma cells has showed 83 significant changes and confirmed up-regulation of Rho/ROCK signalling pathway in highly metastatic A3 sarcoma cells. Increased Rho/ROCK signalling is crucial for amoeboid mode of invasion as the tumour cells with amoeboid mode of invasion do not utilize pericellular degradation of the surrounding extracellular environment (Wolf et al., 2003). By using of Rho-GTPases assay we have also confirmed higher expression of both RhoA-GTP and RhoC-GTP levels in highly metastatic A3 cells, compared to non-metastatic K2 cell line, that activate ROCK (Sahai and Marshall, 2002). Our microarray data have shown significant increased level of caspase-1 that is responsible for degradation of Rho-GDI. According to these findings we have hypothesized that this downregulation of Rho-GDI enables elevated expression of active Rho GTPases in highly invasive A3 cells. To confirm involvement of Rho/ROCK signalling pathway in invasiveness of A3 sarcoma cells, we have established greatly increased of phosphorylated form of MYPT-1 that is the key substrate of ROCK. Conversely, we have observed decreased expression of β-integrin in highly metastatic A3 sarcoma cells. Upregulation of β-integrin is one of the most features of mesenchymally invading cells. These data have pointed out a very interesting fact that the mesenchymal sarcoma A3 cell line has the ability to use

amoeboid mode of invasion, without proteolytic degradation of matrix, which has not been established before.

As it has been reported that the Rho/ROCK signalling pathway regulates actomyosin cytoskeletal dynamics (Amano et al., 1997), our next analyse was focused on measurement of dynamics in K2 and A3 sarcoma cell lines. For these experiments we have used collaboration with the laboratory of Professor Ben Fabry in Erlangen. Using of nanoscale particle tracking methods we have observed elevated dynamics of cortical actin in A3 cell line that is involved in cytoskeleton remodelling and thus can be implicated in higher invasion of A3 cells. These our findings correlated with analysed KINEX microarray data that have shown upregulated expression of cofilin and its phosphorylated form in A3 sarcoma cells. Increased cofilin phosphorylation has led to increased dynamics of F-actin polymerization and depolymerisation and thus to higher cytoskeletal dynamics in highly metastatic A3 cells. We have also shown that F-actin is prominently observed cortically and localized in ruffles at the leading edges in the A3 cells. This arrangement is typical for metastatic cells with higher cytoskeletal dynamics (Veselý et al., 1987), whereas in non-metastatic K2 cells, F- actin was arranged into the stress fibres. These great differences in cytoskeletal dynamics are due to enhanced invasiveness and migration of A3 cells in comparison to K2 cells. We have also confirmed that upregulation of Rho/ROCK signalling pathway in A3 rat sarcoma cells leads to increased phosphorylation of MLC that generates actomyosin traction forces, predominantly localized at the leading edges of migrating cells. Using of Y-27632 inhibitor of ROCK has resulted decreased of invasiveness of A3 sarcoma cells although no changes have been observed in their invasion by using of inhibitor of proteases. Taken together, in this study, we have confirmed for the first time that highly metastatic mesenchymal sarcoma cells can primarily use amoeboid mode of invasion, which is depend on the Rho/ROCK signalling pathway.

Our data have pointed on a very important fact, that many tumours from mesenchymal origin can very effectively use amoeboid mode of invasion via upregulation of the Rho/ROCK signalling pathway. Our findings could signify an important step in cancer therapy of tumour cells using the mesenchymal mode of invasion. As cancer cells are able to undergo mesenchymal-amoeboid transition as an efficient escape from proteases inhibitor cancer therapy, one possibility of how

to prevent tumour cell invasion is the use of treatment based on a combination of ROCK/proteases inhibitors.

Confocal Microscopy Reveals *Myzitiras* and *Vthela* Morphotypes as New Signatures of Malignancy Progression

Neoplastic progression of the human breast cancer cell line G3S1 is associated with elevation of cytoskeletal dynamics and upregulation of MT1-MMP
(the 2nd. and 3rd. publications)

In two other studies, we analysed the role of cytoskeletal dynamics in invasiveness of breast cancer cells. For this study, we used the newly established G3S1 cells, derived from primary human infiltrating EM-G3 ductal breast carcinoma. G3S1 cells have been exposed *in vitro* to chronic nutritional stress by deprivation of specific growth factor and treatments with an activator of protein kinase C (12-O-tetradecanoyl-phorbol-13-acetate).

As neoplastic progression in carcinoma cells usually correlates with enhanced invasiveness of tumour cells through the connective tissues, our data confirmed enhanced invasion of G3S1 cells in comparison to their parental EM-G3 cell lines. Increased invasiveness of G3S1 cells was accompanied with a higher incidence of specific *mysitiras* (sucker-like cell) morphotype. This *mysitiras* morphotype has been characterized by roundish band of adhesion enriched with F-actin and phosphorylated tyrosine residues. The unusual appearance of *mysitiras* morphology could indicate an escape mechanism upon exposure to stressful conditions on the G3S1 cell line, and also an element of epithelial-mesenchymal transition, which is attributing of malignant progression of carcinomas. Elevated invasive potential in G3S1 breast cancer cells correlates with their increased gelatin degradation that has been resulted by activation of matrix metalloproteases. We have shown that more invasive G3S1 cells employ the proteolysis-dependent mode of invasion, which is accompanied by upregulation of MT1-MMP matrix metalloproteases compared to EM-G3 cells.

As accumulation of MT1-MMPs plays a crucial role in invasiveness and metastases, in most tumours (Shiomi et al, 2003), we hypothesized that increased

expression of MT1-MMP in G3S1 could be partially responsible for elevated invasiveness. Although, besides proteolytic degradation of ECM, enhanced dynamics of actin cytoskeleton in G3S1 breast cancer cells facilitates their invasiveness in contrast to EM-G3 cells. Taken together, our results have shown that neoplastic progression and invasiveness of the human breast cancer cell line G3S1 is dependent on increased expression of MT1-MMP matrix metalloprotease and enhanced dynamics of actin cytoskeleton.

NG2-mediated Rho activation promotes amoeboid invasiveness of cancer cells.
(the 4th. publication)

My second project was connected to our previous study from protein microarray analyses KINEX between highly metastatic A3 sarcoma cells and their parental non-metastatic K2 cells. As we surprisingly found a significant decrease in ß1-integrin expression in highly metastatic A3 sarcoma cells, we suggested that ß1-integrin, is not responsible for their amoeboid adhesion. It was recently speculated that amoeboid cell adhesion to the ECM can be mediated via non-integrin receptors, such as glycoproteins (Friedl, 2004). As our previously reported microarray data of A3 and K2 cells have shown highly upregulated levels of NG2 in A3 cells (Cavanna et al., 2007), we have suggested that NG2 proteoglycan could represent the non-integrin ECM receptor of amoeboid cell adhesion. We have analysed the role of NG2 in amoeboid invasiveness, as well as its potential connection in the Rho/ROCK signalling pathway, which is crucial for amoeboid invasion. For our study, we used two independent systems: rat sarcoma K2 and A3 cells; and A375M2 human melanoma cells. We have shown that siRNA-mediated knockdown of NG2 induced amoeboid-mesenchymal transition (AMT) of morphology in both highly metastatic amoeboid cell lines, A3 and A375M2. Both cell lines A3 and A375M2 use amoeboid mode of invasion, thus NG2 inhibition has led to conversion from roundish to elongated morphology, which is typical for mesenchymal mode of invasion. Conversely, overexpression of NG2 proteoglycan has led to mesenchymal-amoeboid transition, increased invasiveness and higher levels of activated Rho in predominantly mesenchymal

K2 cell line. These data suggests the potential of pro-amoeboid role of NG2 proteoglycan. Moreover, knockdown of NG2, in A3 and A375M2, was accompanied with reduced ability of cells to invade through the 3D collagen, as well as great decrease in Rho-GTP expression.

These results have highlighted a previously reported study about potential implementation of NG2 proteoglycan, with Rho/ROCK signalling, in the process of inhibition of neurite outgrowth or promotion of amoeboid invasion. There is an important question of how NG2 could be connected to the Rho/ROCK pathway. We have hypothesized that crucial for this linking is intracellular PDZ binding motif of NG2 proteoglycan that interacts with multivalent PDZ scaffold protein MUPP1 (Barrit et al., 2000). MUPP1 also interacts with Rho-GEF Syx and thus creates the NG2/MUPP1/Syx complex, which promotes localized activation of Rho at the leading edge in migrating cells. (Ernqvist et al., 2009)

The amoeboid-mesenchymal transition in knockdown of A375M2 cells was associated with enhanced ability of the cells to form invadopodia and thus degrade the ECM. However, this enhancement of matrix degradation activity was not a substitution for reduced invasive ability caused by downregulation of Rho/ROCK signaling. Furthermore, overexpression of NG2 proteoglycan in K2 cells has led to decreasing of ECM degradation.

These results have led us to suggest that for the inhibitory effect of NG2 on invadopodia and invasive protrusion formation, a very similar molecular mechanism that has been described in NG2-mediated inhibition of neurite outgrowth could be responsible, and that it is also mediated by Rho/ROCK signalling pathway. Taken together, our data have strongly pointed out the NG2 proteoglycan-mediated invasion via its activation of Rho/ROCK signalling pathway. Moreover, we have suggested that NG2 proteoglycan could serve as a non-integrin adhesive molecule involved in amoeboid cell invasion.

3. CONCLUSIONS

Cancer treatment is very complicated and development of targeted therapies requires the identification of proper targets that are known to play a key role in cancer cell growth and survival, our results imply a few suggestions for new cancer medications and treatments.

Our research focused on the mechanisms of invasion of tumour cells, and was the first to highlight the new finding that highly metastatic sarcoma cells, from mesenchymal origin, primarily use amoeboid mode of invasion, which is mediated by the Rho/ROCK signalling pathway. These data have pointed on the fact that many mesenchymal tumours can promote their invasion via amoeboid type of invasion. Treatment based on combination of both proteases inhibitors and inhibitors against Rho/ROCK pathway could be a good strategy to prevent both amoeboid and mesenchymal type of migration of tumour cells and thus more effectively to block individual cancer cell invasion.

We have also identified NG2 proteoglycan as a potential non-integrin adhesive molecule responsible for amoeboid type of invasion. Treatment directed on NG2 inhibition could prevent the invasion promoted by NG2 proteoglycan whose signalling is potentially connected with Rho/ROCK pathway.

4. REFERENCES

Ahmad, A. and Hart, I. R. (1997). Mechanisms of metastasis. Crit. Rev. Oncol. Hematol. 26, 163-173.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase).. J Biol Chem. 271, 20246-9.

Baldassarre M, Pompeo A, Beznoussenko G, Castaldi C, Cortellino S, McNiven MA, Luini A, Buccione R. (2003).Dynamin participates in focal extracellular matrix degradation by invasive cells. Mol Biol Cell. 14, 1074-84.

Ballestrem C, Hinz B, Imhof BA, Wehrle-Haller B.J (2001).Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. Cell Biol. 155, 1319-32.

Barritt D. S., Pearn M. T., Zisch A. H., Lee S. S., Javier R. T., Pasquale E. B. and Stallcup W. B. (2000). The multi-PDZ domain protein MUPP1 is a cytoplasmic ligand for the membrane-spanning proteoglycan NG2. J Cell Biochem. 79, 213-224.

Behm FG, Raimondi SC, Frestedt JL, Liu Q, Crist WM, Downing JR, Rivera GK, Kersey JH, Pui CH. (1996).Rearrangement of the MLL gene confers a poor prognosis in childhood acute lymphoblastic leukemia, regardless of presenting age. Blood. 87, 2870-7.

Benassi MS, Pazzaglia L, Chiechi A, Alberghini M, Conti A, Cattaruzza S, Wassermann B, Picci P, Perris R. (2009). NG2 expression predicts the metastasis formation in soft-tissue sarcoma patients. J Orthop Res. 27,135-40.

Bertini I, Fragai M, Luchinat C, Melikian M, Venturi C.(2009). Characterisation of the MMP-12-elastin adduct. Chemistry. 15, 7842-5

Bigg HF, Shi YE, Liu YE, Steffensen B, Overall CM. (1997). Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A. TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. J Biol Chem 272, 15496-500.

Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW. (2002).The organizing principle: microenvironmental inXuences in the normal and malignant breast. Di Verentiation. 70, 537–546

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W. (2003). Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. Mol Cell Neurosci. 22, 405-16.

Brábek J, Mierke CT, Rösel D, Veselý P, Fabry B (2010). The role of the tissue microenvironment in the regulation of cancer cell motility and invasion..Cell Commun Signal. 7, 8-22.

Burg M. A., Tillet E., Timpl R. and Stallcup W. B. (1996). Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. J Biol Chem. 271, 26110-26116.

Burg MA, Nishiyama A, Stallcup WB. (1997). A central segment of the NG2 proteoglycan is critical for the ability of glioma cells to bind and migrate toward type VI collagen. Exp Cell Res. 235, 254-64

Burg MA, Grako KA, Stallcup WB.(1998). Expression of the NG2 proteoglycan enhances the growth and metastatic properties of melanoma cells. J Cell Physiol. 177, 299-312

Burg MA, Pasqualini R, Arap W, Ruoslahti E, Stallcup WB. (1999). NG2 proteoglycan-binding peptides target tumor neovasculature. Cancer Res. 59, 2869-74

Carr J, Carr I, Dreher B, Betts K.J (1980). Lymphatic metastasis: invasion of lymphatic vessels and efflux of tumour cells in the afferent popliteal lymph as seen in the Walker rat carcinoma. Pathol. 132, 287-305.

Cattaruzza S, Nicolosi PA, Perris R. (2008). Proteoglycans in the control of tumor growth and metastasis formation.Connect Tissue Res. 49, 225-9.

Cavanna T, Pokorná E, Veselý P, Gray C, Zicha D. (2007). Evidence for protein 4.1B acting as a metastasis suppressor J. Cell Sci. 120,606–616

Celniker S. E., Wheeler D. A., Kronmiller B., Carlson J. W., Halpern A., Patel S., Adams M., Champe M., Dugan S. P., Frise E., Hodgson A., George R. A., Hoskins R. A., Laverty T., Muzny D. M., Nelson C. R., Pacleb J. M., Park S., Pfeiffer B. D., Richards S., Sodergren E. J., Svirskas R., Tabor P. E., Wan K., Stapleton M., Sutton G. G., Venter C., Weinstock G., Scherer S. E., Myers E. W., Gibbs R. A. and Rubin G. M. (2002). Finishing a whole-genome shotgun: release 3 of the Drosophila melanogaster euchromatic genome sequence. Genome Biol 3, RESEARCH0079.

Charras GT, Yarrow JC, Horton MA, Mahadevan L, Mitchison TJ. (2005). Non-equilibration of hydrostatic pressure in blebbing cells. Nature. 435, 365-9.

Charras G, Paluch E. (2008). Blebs lead the way: how to migrate without lamellipodia.Nat Rev Mol Cell Biol. 9, 730-6. Review.

Chatterjee N, Stegmüller J, Schätzle P, Karram K, Koroll M, Werner HB, Nave KA, Trotter J.(2008) Interaction of syntenin-1 and the NG2 proteoglycan in migratory oligodendrocyte precursor cells. J Biol Chem. 283, 8310-7.

Chekenya M, Pilkington GJ. (2002). NG2 precursor cells in neoplasia: functional,

histogenesis and therapeutic implications for malignant brain tumours.J Neurocytol. 31, 507-521.

Chekenya M, Enger PØ, Thorsen F, Tysnes BB, Al-Sarraj S, Read TA, Furmanek T, Mahesparan R, Levine JM, Butt AM, Pilkington GJ, Bjerkvig R. (2002).The glial precursor proteoglycan, NG2, is expressed on tumour neovasculature by vascular pericytes in human malignant brain tumours.Neuropathol Appl Neurobiol. 28, 367-80.

Chu ML, Mann K, Deutzmann R, Pribula-Conway D, Hsu-Chen CC, Bernard MP, Timpl R. (1987). Characterization of three constituent chains of collagen type VI by peptide sequences and cDNA clones. Eur J Biochem. 168, 309-17.

Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol. 3, 339-45.

Deryugina EI, Bourdon MA, Reisfeld RA, Strongin A. (1998). Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2.. Cancer Res. 58, 3743-50.

Dou C. L. and Levine J. M. (1994). Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. J Neurosci. 14, 7616-7628.

Dou CL, Levine JM. (1997). Identification of a neuronal cell surface receptor for a growth inhibitory chondroitin sulfate proteoglycan NG2. J Neurochem. 68, 1021-30.

Douglas DA, Shi YE, Sang QA. (1997). Computational sequence analysis of the tissue inhibitor of metalloproteinase family. J Protein Chem.16, 237-55.

Eisenmann K. M., McCarthy J. B., Simpson M. A., Keely P. J., Guan J. L., Tachibana K., Lim L., Manser E., Furcht L. T. and Iida J. (1999). Melanoma chondroitin sulphate proteoglycan regulates cell spreading through Cdc42, Ack-1 and p130cas. Nat Cell Biol. 1, 507-513.

Ernkvist M, Luna Persson N, Audebert S, Lecine P, Sinha I, Liu M, Schlueter M, Horowitz A, Aase K, Weide T, Borg JP, Majumdar A, Holmgren L.(2009). The Amot/Patj/Syx signaling complex spatially controls RhoA GTPase activity in migrating endothelial cells. Blood. 113, 244-53.

Fackler OT, Grosse R. J. (2008). Cell motility through plasma membrane blebbing. Cell Biol 181, 879-84. Review

Fang X., Burg M. A., Barritt D., Dahlin-Huppe K., Nishiyama A. and Stallcup W. B. (1999). Cytoskeletal reorganization induced by engagement of the NG2 proteoglycan leads to cell spreading and migration. Mol Biol Cell. 10, 3373-3387.

Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A, Mueller BM.(2001).

Integrin activation controls metastasis in human breast cancer. Proc Natl Acad Sci U S A. 98, 1853-8.

Folkman J. (1972). Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg.175, 409-16.

Fournier AE, Takizawa BT, Strittmatter SM. (2003). Rho kinase inhibition enhances axonal regeneration in the injured CNS.J Neurosci. 23, 1416-23.

Friedl P, Zanker KS, Bro¨ cker E-B. (1998). Cell migration strategies in 3D extracellular matrix: differences in morphology, cell matrix interactions, and integrin function. Microsc Res Tech 43,369-378.

Friedl P. and Wolf K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 3: 362-374.

Friedl P. (2004). Prespecification and plasticity: shifting mechanisms of cell migration. Curr. Opin. Cell Biol. 16: 14-23.

Friedl P, Wolf K. (2009). Proteolytic interstitial cell migration: a five-step process. Cancer Metastasis Rev. 28, 129-35. Review.

Fukushi J., Makagiansar I. T. and Stallcup W. B. (2004). NG2 proteoglycan promotes endothelial cell motility and angiogenesis via engagement of galectin-3 and alpha3beta1 integrin. Mol Biol Cell. 15, 3580-3590.

Garrigues HJ, Lark MW, Lara S, Hellstrom I, Hellstrom KE, Wight TN. (1986). The melanoma proteoglycan: restricted expression on microspikes, a specific microdomain of the cell surface. J Cell Bio.l 103, 1699-1710.

Gates MA, Fillmore H, Steindler DA (1996). Chondroitin sulfate proteoglycan and tenascin in the wounded adult mouse neostriatum in vitro: dopamine neuron attachment and process outgrowth. J Neurosci. 16, 8005-18.

Grako KA, Stallcup WB. (1995). Participation of the NG2 proteoglycan in rat aortic smooth muscle cell responses to platelet-derived growth factor. Exp. Cell res.221,231-40.

Grinnell FJ. (1994). Fibroblasts, myofibroblasts, and wound contraction. Cell Biol. 124, 401-4
Hanahan D, Weinberg RA. (2000). The hallmarks of cancer. Cell. 100, 57–70

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ.(1999). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF..Science. 284, 1994-8.

Hulit J, Suyama K, Chung S, Keren R, Agiostratidou G, Shan W, Dong X, Williams TM, Lisanti MP, Knudsen K, Hazan RB (2007). N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation.Cancer Res. 7, 3106-3116.

Hyun-Jeong Ra and William C. Parks (2007). Control of Matrix Metalloproteinase Catalytic Activity. Matrix Biol, 26, 587–596.

Iida J., Meijne A. M., Spiro R. C., Roos E., Furcht L. T. and McCarthy J. B. (1995). Spreading and focal contact formation of human melanoma cells in response to the stimulation of both melanoma-associated proteoglycan (NG2) and alpha 4 beta 1 integrin. Cancer Res. 55, 2177-2185.

Iida J., Meijne A. M., Oegema T. R., Jr., Yednock T. A., Kovach N. L., Furcht L. T. and McCarthy J. B. (1998). A role of chondroitin sulfate glycosaminoglycan binding site in alpha4beta1 integrin-mediated melanoma cell adhesion. J Biol Chem 273, 5955-5962.

Iida J., Pei D., Kang T., Simpson M. A., Herlyn M., Furcht L. T. and McCarthy J. B. (2001). Melanoma chondroitin sulfate proteoglycan regulates matrix metalloproteinase-dependent human melanoma invasion into type I collagen. J Biol Chem. 276, 18786-94.

Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt SM, Helman LJ. (2004). The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis Nat Med 10, 182-6.

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K.(1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase) Science. 273, 245-8.

Krüger A, Soeltl R, Sopov I, Kopitz C, Arlt M, Magdolen V, Harbeck N, Gänsbacher B, Schmitt M. (2001).Hydroxamate-type matrix metalloproteinase inhibitor batimastat promotes liver metastasis.Cancer Res. 61, 1272-5.

Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R, Hirsch K, Keller M, Förster R, Critchley DR, Fässler R, Sixt M. (2008).Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature. 455, 51-5.

Lauffenburger D.A. and Horwitz A.F. (1996). Cell migration: a physically integrated molecular process. Cell 84: 359-369.

Levine J. M. (1994). Increased expression of the NG2 chondroitin-sulfateproteoglycan after brain injury. J Neurosci. 14, 4716-4730.

Lin X. H., Dahlin-Huppe K. and Stallcup W. B. (1996). Interaction of the NG2 proteoglycan with the actin cytoskeleton. J Cell Biochem. 63, 463-477.

Lin X. H., Grako K. A., Burg M. A. and Stallcup W. B. (1996). NG2 proteoglycan and the actin-binding protein fascin define separate populations of actin-containing filopodia and lamellipodia during cell spreading and migration. Mol Biol Cell. 7, 1977-1993.

Liu FT, Rabinovich GA. (2005). Galectins as modulators of tumour progression. Nat Rev Cancer. 5,29-41

Lubbe WJ, Zhou ZY, Fu W, Zuzga D, Schulz S, Fridman R, Muschel RJ, Waldman SA, Pitari GM.. (2006). Tumor epithelial cell matrix metalloproteinase 9 is a target for antimetastatic therapy in colorectal cancer. Clin Cancer Res 12,1876-82.

Majumdar M., Vuori K. and Stallcup W. B. (2003). Engagement of the NG2 proteoglycan triggers cell spreading via rac and p130cas. Cell Signal. 15, 79-84.

Makagiansar I. T., Williams S., Dahlin-Huppe K., Fukushi J. I., Mustelin T. and Stallcup W. B. (2004). Phosphorylation of NG2 proteoglycan by PKC-alpha regulates polarized membrane distribution and cell motility. J Biol Chem. 279, 55262-70

Makagiansar IT, Williams S, Mustelin T, Stallcup WB. (2007). Differential phosphorylation of NG2 proteoglycan by ERK and PKCalpha helps balance cell proliferation and migration.J Cell Biol. 178, 155-65.

Mauvieux L, Delabesse E, Bourquelot P, Radford-Weiss I, Bennaceur A, Flandrin G, Valensi F, MacIntyre EA. (1999). NG2 expression in MLL rearranged acute myeloid leukaemia is restricted to monoblastic cases. Br J Haematol. 107, 674-6.

McKeon RJ, Schreiber RC, Rudge JS, Silver J. (1991).Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J Neurosci. 11,3398-411

Miki H, Takenawa T.J (2003). Regulation of actin dynamics by WASP family proteins. Biochem. 134, 309-13. Review

Millard TH, Sharp SJ, Machesky LM . (2004). Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex.Biochem J. 380, 1-17. Review

Monaco S, Gioia M, Rodriguez J, Fasciglione GF, Di Pierro D, Lupidi G, Krippahl L, Marini S, Coletta M. (2007).Modulation of the proteolytic activity of matrix metalloproteinase-2 (gelatinase A) on fibrinogen. Biochem J. 402, 503-13.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. (2003). The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci. 22,319-30.

Nagase H, Visse R, Murphy G. (2006).Structure and function of matrix metalloproteinases and TIMPs.. Cardiovasc Res. 69, 562-73.

Nannuru KC, Futakuchi M, Varney ML, Vincent TM, Marcusson EG, Singh RK. (2010). Matrix metalloproteinase (MMP)-13 regulates mammary tumor-induced

osteolysis by activating MMP9 and transforming growth factor-beta signaling at the tumor-bone interface. Cancer Res. 70, 3494-504.

Ng T., Parsons M., Hughes W. E., Monypenny J., Zicha D., Gautreau A., Arpin M., Gschmeissner S., Verveer P. J., Bastiaens P. I. and Parker P. J. (2001). Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. Embo J. 20, 2723-2741

Niehaus A., Stegmuller J., Diers-Fenger M. and Trotter J. (1999). Cell-surface glycoprotein of oligodendrocyte progenitors involved in migration. J Neurosci. 19, 4948-4961.

Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ (1999). N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression..J Cell Biol. 147, 631-44.

Nishiyama A., Dahlin K. J., Prince J. T., Johnstone S. R. and Stallcup W. B. (1991). The primary structure of NG2, a novel membrane-spanning proteoglycan. J Cell Biol. 114, 359-371.

Nishiyama A., Lin X. H. and Stallcup W. B. (1995). Generation of truncated forms of the NG2 proteoglycan by cell surface proteolysis. Mol Biol Cell. 6, 1819-1832.

Nobes CD, Hall A. (1995).Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 81,53-62.

Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, Proost P, Van Damme J.J (2001).Gelatinase B functions as regulator and effector in leukocyte biology. Leukoc Biol. 69, 851-9. Review.

Overall CM, Kleifeld O. (2006). Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nat Rev Cancer. 6,227-39.

Paňková D, Jobe N, Kratochvílová M, Buccione R, Brábek J, Rösel D. (2012). NG2-mediated Rho activation promotes amoeboid invasiveness of cancer cells.Eur J Cell Biol. 2012

Perris R, Johansson S. (1987). Amphibian neural crest cell migration on purified extracellular matrix components: a chondroitin sulfate proteoglycan inhibits locomotion on fibronectin substrates. J Cell Biol. 105, 2511-21.

Piccard H, Van den Steen PE, Opdenakker G (2007). Hemopexin domains as multifunctional liganding modules in matrix metalloproteinases and other proteins. J Leukoc Biol. 2007 81, 870-92.

Pluschke G., Vanek M., Evans A., Dittmar T., Schmid P., Itin P., Filardo E. J. and

Reisfeld R. A. (1996). Molecular cloning of a human melanoma-associated chondroitin sulfate proteoglycan. Proc Natl Acad Sci. U S A 93, 9710-9715.

Poincloux R, Lizárraga F, Chavrier P. (2009). Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia. J Cell Sci. 122, 3015-24. Review.

Provenzano PP, Inman DR, Eliceiri KW, Trier SM, Keely PJ. (2008). Contact guidance mediated three-dimensional cell migration is regulated by Rho/ROCK-dependent matrix reorganization. Biophys J. 95, 5374-84

Rapraeger A, Jalkanen M, Bernfield M. (1986). Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. J Cell Biol. 103, 2683-96.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.. Cell. 70, 401-10.

Roeb E, Schleinkofer K, Kernebeck T, Pötsch S, Jansen B, Behrmann I, Matern S, Grötzinger J (2002). The matrix metalloproteinase 9 (mmp-9) hemopexin domain is a novel gelatin binding domain and acts as an antagonist..J Biol Chem. 277, 50326-32.

Rösel D, Brábek J, Tolde O, Mierke CT, Zitterbart DP, Raupach C, Bicanová K, Kollmannsberger P, Panková D, Vesely P, Folk P, Fabry B.. (2008) Up-regulation of Rho/ROCK signaling in sarcoma cells drives invasion and increased generation of protrusive forces. Mol Cancer Res. 6, 1410-20

Sabeh F, Shimizu-Hirota R, Weiss SJ. (2009). Protease-dependent versus -independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. J Cell Biol. 185, 11-9.

Sahai E, Marshall CJ. (2003). Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis.. Nat Cell Biol. 5, 711-9.

Sahai E. (2005). Mechanisms of cancer cell invasion. Curr Opin Genet Dev. 15,87-96. Review

Schlingemann RO, Rietveld FJ, de Waal RM, Ferrone S, Ruiter DJ.(1990). Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. Am J Pathol. 36, 1393-405.

Schlingemann RO, Oosterwijk E, Wesseling P, Rietveld FJ, Ruiter DJ. (1996). Aminopeptidase a is a constituent of activated pericytes in angiogenesis.J Pathol. 179, 436-42.

Sells MA, Boyd JT, Chernoff J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts.J Cell Biol. 145, 837-49.

Shoshan Y, Nishiyama A, Chang A, Mörk S, Barnett GH, Cowell JK, Trapp BD, Staugaitis SM. (1999).Expression of oligodendrocyte progenitor cell antigens by gliomas: implications for the histogenesis of brain tumors. Proc Natl Acad Sci U S A. 96, 10361-6.

Smith A, Bracke M, Leitinger B, Porter JC, Hogg N. (2003). LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. J Cell Sci.116, 3123-33.

Sohail A, Sun Q, Zhao H, Bernardo MM, Cho JA, Fridman R. (2008). MT4-(MMP17) and MT6-MMP (MMP25), A unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer. Cancer Metastasis Rev. 27, 289-302. Review.

Stallcup W. B. (1981). The NG2 antigen, a putative lineage marker: immunofluorescent localization in primary cultures of rat brain. Dev Biol. 83, 154-165.

Stallcup W. B., Dahlin K. and Healy P. (1990). Interaction of the NG2 chondroitin sulfate proteoglycan with type VI collagen. J Cell Biol. 111, 3177-3188.

Stallcup W. B. and Dahlin-Huppe K. (2001). Chondroitin sulfate and cytoplasmic domain-dependent membrane targeting of the NG2 proteoglycan promotes retraction fiber formation and cell polarization. J Cell Sci. 114, 2315-2325.

Stallcup W. B. (2002). The NG2 proteoglycan: past insights and future prospects. J Neurocytol. 31, 423-435.

Stallcup W.B, Huang F.J. (2008). A role for the NG2 proteoglycan in glioma progression. Cell Adh. Migr. 2, 192–201

Stallcup WB. (2008). The progenitor cell marker NG2/MPG promotes chemoresistance by activation of integrin-dependent PI3K/Akt signaling. Oncogene. 27,5182-94.

Stegmuller J., Schneider S., Hellwig A., Garwood J. and Trotter J. (2002). AN2, the mouse homologue of NG2, is a surface antigen on glial precursor cells implicated in control of cell migration. J Neurocytol. 31, 497-505.

Stegmuller J., Werner H., Nave K. A. and Trotter J. (2003). The proteoglycan NG2 is complexed with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors by the PDZ glutamate receptor interaction protein (GRIP) in glial progenitor cells. Implications for glial-neuronal signaling. J Biol Chem . 278, 3590-3598.

Stetler-Stevenson, W.G., S. Aznavoorian, and L.A. Liotta. (1993). Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu. Rev. Cell Biol. 9,541–573.

Tallant C, Marrero A, Gomis-Rüth FX. (2010). Matrix metalloproteinases: fold and function of their catalytic domains. Biochim Biophys Acta.1803, 20-8. Review.

Tamariz E, Grinnell F (2002)Modulation of fibroblast morphology and adhesion during collagen matrix remodeling.Mol Biol Cell. 13, 3915-29.

Tillet E, Ruggiero F, Nishiyama A, Stallcup W.B. (1997), The membrane-spanning proteoglycan NG2 binds to collagens V and VI through the central nonglobular domain of its core protein J. Biol. Chem., 272,769–776

Tillet E, Gential B., Garrone G, Stallcup W.B. (2002) NG2 proteoglycan mediates beta1 integrin-independent cell adhesion and spreading on collagen VI J. Cell. Biochem. 86,726–736

Thiery JP. (2002). Epithelial-mesenchymal transitions in tumour progression.Nat Rev Cancer. 2, 442-54. Review.

Trotter J, Karram K, Nishiyama A. (2010). NG2 cells: Properties, progeny and origin. Brain Research Rev. 63,72-82).

Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, Debruyne FM, Schalken JA.(1994). Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res. 54, 3929-33.

Visse R, Nagase H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 92, 827-39. Review.

Wang H, Nawata J, Kakudo N, Sugimura K, Suzuki J, Sakuma M, Ikeda J, Shirato K. J Hypertens. (2004). The upregulation of ICAM-1 and P-selectin requires high blood pressure but not circulating renin-angiotensin system in vivo. 22, 1323-32

Wang X, Wang Y, Yu L, Sakakura K, Visus C, Schwab J.H, Ferrone C.R, Favoino E, Y. Koya, M.R. Campoli, J.B. McCarthy, A.B. DeLeo, S. Ferrone (2010). CSPG4 in cancer: multiple roles Curr. Mol. Med. 10,419–429

Watanabe M, Takahashi Y, Ohta T, Mai M, Sasaki T, Seiki M. (1996). Inhibition of metastasis in. human gastric cancer cells transfected with tissue inhibitor of metalloproteinase 1 gene in nude mice. Cancer. 77, 1676-80.

Watanabe, T., Noritake, J. and Kaibuchi, K. (2005) Regulation of microtubules in cell migration. Trends Cell Biol. 15, 76–83

Wen Y, Makagiansar IT, Fukushi J, Liu FT, Fukuda MN, Stallcup WB. (2006). Molecular basis of interaction between NG2 proteoglycan and galectin-3. J Cell Biochem. 98, 115-27.

Werr J, Xie X, Hedqvist P, Ruoslahti E, Lindbom L. (1998). Beta1 integrins are critically involved in neutrophil locomotion in extravascular tissue In vivo. J Exp Med. 187, 2091-6.

Wolf K, Müller R, Borgmann S, Bröcker EB, Friedl P. (2003) Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. Blood. 102, 3262-9.

Worthylake RA, Lemoine S, Watson JM, Burridge K. (2001). RhoA is required for monocyte tail retraction during transendothelial migration. J Cell Biol. 54, 147-60.

Wyckoff JB, Segall JE, Condeelis JS. (2000). The collection of the motile population of cells from a living tumor. Cancer Res. 60, 5401-4.

Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res. 64, 7022-9.

Wyckoff JB, Pinner SE, Gschmeissner S, Condeelis JS, Sahai E. (2006).ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. Curr Biol. 16, 1515-23.

Xu D, McKee CM, Cao Y, Ding Y, Kessler BM, Muschel RJ (2010). Matrix metalloproteinase-9 regulates tumor cell invasion through cleavage of protease nexin-1.. Cancer Res. 70, 6988-98.

Yamagata M, Suzuki S, Akiyama SK, Yamada KM, Kimata K. (1989). Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. J Biol Chem. 264, 8012-8.

Yamaguchi Y, Ruoslahti E. (1988). Expression of human proteoglycan in Chinese hamster ovary cells inhibits cell proliferation. Nature. 336, 244-6.

Yanai M, Kenyon CM, Butler JP, Macklem PT, Kelly SM.(1996). Intracellular pressure is a motive force for cell motion in Amoeba proteus. Cell Motil Cytoskeleton. 33, 22-9.

Zucker S, Cao J, Chen WT. (2000). Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. Oncogene. 19, 6642-50. Review.

5. SUPPLEMENTARY DATA