CHARLES UNIVERSITY IN PRAGUE
Faculty of Science
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Bachelor´s thesis

THE ROLE OF NG2 GLYCOPROTEIN IN CANCER CELL INVASIVENESS

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I would like to thank to my supervisor RNDr. Jan Brábek, Ph.D. for his helpful advices and the time he dedicated to me. Also I would like to thank to Mgr. Daniela Paňková for her stimulating ideas.

I declare that I have my thesis “The role of NG2 glycoprotein in cancer cell invasiveness” prepared on my own, based only on the cited literature and on consultations with my supervisor.

Prague 2010 Adam Obr

ABSTRACT

NG2 proteglycan is a novel membrane – spanning proteoglycan, expressed in general in developing tissue whose cells are characteristic for its increased level of proliferation and motility. NG2 proteoglycan is considered to be an anchor for cell adhesion capabilities on different substrata as well as a signaling transmembrane structure which is capable of affecting actin cytoskeleton and causing increased cell migration. This bibliographic search shows the considered effect of NG2 proteoglycan to the migration abilities of cancer cells via different molecular mechanisms, such as NG2 – mediated, integrin – independent cell interactions with collagens and other ECM substrata, effect of phosphorylation with two different kinases leading to diverse signaling and different behavior in response to phosphorylation and finally the interaction with scaffolding protein MUPP1 and possible connection with signaling pathway to RhoA GTPase, which is involved in cytoskeleton regulation.

Keywords:

NG2 proteoglycan, MCSP, cancer invasiveness, cell motility, cell adhesion, differential phosphorylation, transmembrane signaling

ABSTRAKT

NG2 proteoglykan je jedinečný transmembránový glykoprotein, který je exprimován hlavně vyvíjející se tkání, jejíž buňky jsou charakteristické zvýšenou úrovní proliferace a migrační schopností. NG2 proteoglykan je považován jednak za zprostředkovatele buněčné adheze na různých substrátech, jednak za signální transmembránovou strukturu, která je schopná ovlivňovat aktinový cytoskelet a způsobovat zvýšenou úroveň pohyblivosti buňky. Tato literární rešerše poukazuje na možný efekt NG2 glykoproteinu na migrační schopnosti nádorových buněk skrze různé molekulární mechanismy, jako je na integrinech nezávislá interakce buňky s kolageny a dalšími substráty mimobuněčné matrice, efekt fosforylace dvěmi odlišnými kinázami vedoucí k odlišné signalizaci a různému chování v odpovědi na onu fosforylaci, a konečně interakci se scaffold proteinem MUPP1 a jeho možným spojením se signální drahou vedoucí k RhoA GTPáze, která je zapojená do regulací cytoskeletu.

Klíčová slova:

NG2 proteoglykan, MCSP, nádorová invazivita, buněčná hybnost, buněčná adheze, diferenciální fosforylace, transmembránová signalizace

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INDEX OF ABBREVIATIONS

cDNA	complementary DNA
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
Cys	cysteine
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ERK	extracellular signal – regulated kinase
GEF	G – nucleotide exchange factor
HMW – MAA	high molecular weight melanoma associated antigen
MAEC	mouse aortic endothelial cell
MCSP	melanoma chondroitin sulfate proteoglycan
mRNA	messenger RNA
NG2	nerve – glial antigen 2
PDGF –BB	platelet – derived growth factor BB
PDGFR α	platelet – derived growth factor receptor alpha
PKC α	protein kinase C alpha
Thr	threonine

INTRODUCTION

Cancer is one of the world most frequent causes of death. Its dangerousness results not only from uncontrolled cell growth, but also from the ability of tumor cells to spread throughout the organism. The spreading occurs mainly in two different manners, via the mesenchymal mechanism, when the cell is able to express proteases and simply decompose surrounding tissue in order to make space for migration, or, on the other hand, the amoeboid mechanism, when the cell is able to adhere to the tissue and via the adaptability of cytoskeleton is capable of moving through without harming the surrounding tissue (Wolf et al. 2003, Sahai and Marshall 2003). Understanding the molecular basis of those mechanisms is crucial for us to fight effectively the spreading of cancer throughout the organism. NG2 proteoglycan seems to play a significant role in the cancer cells behavior. This work will concentrate on the role of NG2 proteoglycan in cancer cell invasiveness, in processes which are involved in cell adhesion and cell motility and results in uncontrolled cell spreading throughout the whole system.

NG2 DISCOVERY

Proteoglycans ale molecules containing glycosaminoglycan chains covalently linked to the core proteins. Many of these molecules are found in the extracellular matrix or on the cell surface and have been implicated in a wide variety of cell functions, such as cell adhesion, cell migration, and proliferation (Nishiyama et al. 1991).

The Nerve – Glial Antigen 2, also known as NG2 chondroitin sulfate proteoglycan, is a rat homologue of the human melanoma proteoglycan, (melanoma chondrotin sulphate proteoglycan, MCSP) and the mouse AN2 protein (Stallcup, Beasley and Levine 1983, Niehaus et al. 1999; according to Stallcup 2002).

First time the NG2 was discovered in 1982 as „high molecular weight melanoma – associated antigen", HMW – MAA (Houghton et al. 1982; according to Stallcup 2002). A year later, 1983, the structure was described in neurons and named „Nerve – glial antigen 2", NG2 (Stallcup et al. 1983). There was a protein called „human melanoma proteoglycan" as well, and after publishing a complete cDNA and amino acid sequence in year 1991, comparison of the amino – terminal sequences showed clearly that human melanoma proteoglycan is the human homologue of NG2 (Nishiyama et al. 1991). Since its discovery in neural cells, there were described many of its activities in cell regeneration, proliferation and motility, and its participation in pathological processes as well.

NG2 EXPRESSION

Generally, expression of NG2 is widespread in a developing tissue, which is characteristic for its increased cell proliferation and motility. NG2 is not expressed in multipotent stem cells but it is upregulated once stem cell makes an initial commitment to a particular cellular lineage. The strongest expression of NG2 can be found on partially-committed progenitor cells that are still proliferative, motile and retain a certain degree of developmental plasticity. Upon terminal differentiation of these progenitors, NG2 expression is downregulated. Important is that NG2 expression is upregulated in many types of injury and pathological situations, including tumors that are characterized by renewed cell proliferation and motility. This suggests that the NG2 proteoglycan should serve as a marker for an "activated" status of cells (Stallcup and Huang 2008).

In central nervous system, NG2 expression is largely restricted to oligodendrocyte progenitor cells and the only exception seems to be pericytes associated with the central nervous system vasculature. In conjunction with PDGFRα, NG2 has become a reliable marker for these progenitors (Dawson et al. 2003; according to Stallcup and Huang 2008). After completion of myelination process, large numbers of NG2-positive and PDGFRα – positive cells persist in the adult brain and spinal cord. This could mean that the NG2 – positive and PDGFRα – positive cells represent a pool of persistent oligodendrocyte progenitors, or a third class of differentiated microglia with yet unknown functions. From the standpoint of cancer research, the key observation is that the adult central nervous system contains a large number of cycling NG2 – positive progenitors, which are potentially vulnerable to transformation (Stallcup and Huang 2008).

The experiments suggest that NG2, or chondroitin sulfate proteoglycans in general, are inhibitory to the axonal outgrowth when CNS is wounded. Axons fail to regenerate in central nervous system following injury and this lack of regeneration appears to be due to nonpermissive properties of CNS environment (Monnier et al. 2003). Numerous studies demonstrate that chondroitin sulfate proteoglycans can inhibit neurite outgrowth of various neuronal cell types. Additionally, Bradbury et al. demonstrated that chondroitinase treatment supports regeneration (Bradbury et al. 2002), and according to Monnier et al. inhibition of Rho GTPase by C3 transferase and inhibition of Rho kinase (ROCK) by Y27632 abolishes the inhibitory effect of CSPGs, probably due to the signal connection between CSPGs and Rho/ROCK signalization pathway (Monnier et al. 2003). The question is why NG2 seems to be the activator of healing and repairing of tissue by cell division and motility and, on the other hand, it inactivates the growth of cellular structures and in fact restrains the correct healing of CNS tissue (figure 1). As a matter of fact, it should be noted that the neurons used for the *in vitro* models are from neonatal animals. There is a difference in molecular expression patterns in adult CNS and neonatal CNS and these differences could affect the impact of NG2 on axonal outgrowth. Secondly, the NG2 itself is unlikely to be a major inhibitor of axonal regeneration after injury to the CNS and, further, that NG2 is unlikely to be necessary for regeneration or functional recovery following peripheral nerve injury (Hossain-Ibrahim et al. 2007).

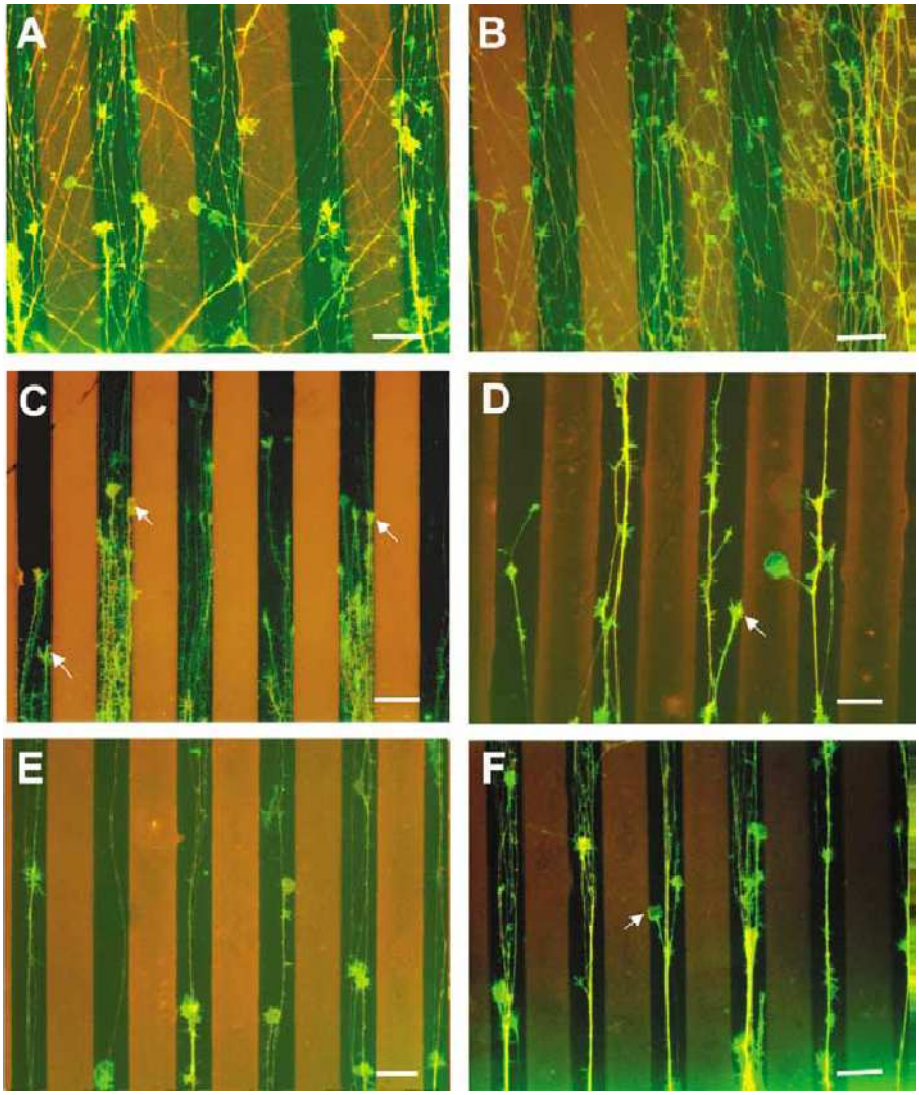

Fig. 1: **THE NEURITE OUTGROWTH INHIBITORY EFFECT OF PROTEOGLYCANS**

The neurite outgrowth inhibitory effect of proteoglycans on axons is concentration dependent. Temporal (A, C, and E) and nasal (B, D, and F) retina explants were cultured on substrates patterned with alternating stripes of laminin and laminin plus chondroitin sulfate proteoglycans (CSPGs). When grown on stripes alternating laminin and laminin plus 5 µg/ml CSPGs (A and B), temporal and nasal axons did not show any clear preference. When grown on substrates with alternating laminin and laminin/CSPG (10 µg/ml CSPGs; C and D) or laminin/CSPG (20 µg/ml CSPGs; E and F), temporal and nasal axons clearly avoided the CSPG - containing stripes. The arrows mark growth cones that contact CSPG stripes.
Bar = 60 µm.

Adapted from Monnier et al.: The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar (2003)

EXPRESSION OUTSIDE THE CENTRAL NERVOUS SYSTEM

The NG2 proteoglycan has a widespread distribution also in many other tissues outside the central nervous system. Huge upregulation is observed for example in mesenchymal cells that represent immature chondroblasts. Again, as chondroblasts differentiate into chondrocytes, NG2 expression is largely downregulated. The similar pattern is observed during osteoblast to osteocyte transition on a maturing bone. In developing skin, NG2 is expressed by keratinocyte progenitors derived from NG2-negative keratinocyte stem cells. The proteoglycan is also upregulated in developing vasculature, particularly in vascular mural cells, in the developing heart on cardiomyocytes, on smooth muscle cells and on pericytes in microvessels. As we can see, expression of NG2 follows the pattern of upregulation in growing or wound repairing tissue (Stallcup and Huang 2008).

NG2 EXPRESSION AT PATHOLOGICAL CONDITIONS

In adult animals, the expression of NG2 is restricted to tumor cells and angiogenic tumor vasculature, making this proteoglycan a potential target for directing therapeutic agents to relevant sites of action. NG2 is highly expressed in melanomas, gliomas, chondrosarcomas and lymphoid leukemias. As we can see, there is an obvious relation of natural presence of NG2, mentioned above, and pathological state. Cancer cells are keeping high expression levels of NG2, as it can be observed in natural occurrence of "activated" cell status. This would be consistent with ideas concerning the ability of NG2 to potentiate cell motility and cell proliferation in response to stimulation by growth factors and extracellular matrix components. Several reports suggest that expression of the proteoglycan is correlated with the degree of malignancy of the glioma and other types of cancer. In fact it is considered whether NG2 could serve as a marker for invasive cancer cells as well as it can serve along with PDGFRα for progenitors in central nervous system. (Chekenya and Pilkington 2002; according to Stallcup and Huang 2008)

NG2 is also expressed by pericytes in tumor microvasculature. Studies have reported the frequent occurrence in tumors of NG2 positive segments of blood vessels that are devoid of endothelial cells. This reflects the disregulation of pericyte – endothelial cell interaction under pathological conditions. There were also reports of robust expression of NG2 in glioma vasculature and expression of NG2 by glioma cells themselves has been noted to have important effects on the characteristics of the glioma vasculature. (Stallcup and Huang 2008)

STRUCTURE OF NG2 PROTEOGLYCAN

The structure of NG2, a large chondroitin sulfate proteoglycan, was first time predicted in 1991. The primary sequence was determined from cDNA clones, which hybridize to an mRNA of 8,9 kbp from rat neural cell lines. The total cDNA contains 8071 nucleotides and determines an open reading frame for 2325 amino acids, calculated molecular mass for NG2 core protein is 251,47 Da. (Nishiyama et al. 1991)

The core protein is synthetized as a 260 kDa peptide, and then posttranslationally processed to a 300 kDa glycoprotein. It contains 14 potential N – linked glycosylation sites, but there is no evidence which ones are really used. The core is composed by 2224 amino acid extracellular domain, 25 amino acid transmembrane domain (residues 2225-2249, followed by several basic residues) and 76 amino acid cytoplasmic domain. (Nishiyama et al. 1991)

THE EXTRACELLULAR PART OF NG2

In general, the extracellular part of NG2 is composed of two globular domains, which are connected with the central segment. The first of the extracellular domains is a N – terminal, cysteine rich domain (residues 30 – 640) containing eight Cys residues. The relative positions and the intervening amino acid sequences of these residues are significantly different from those found in other extracellular and cell surface molecules, such as EGF – like repeats and immunoglobulin – like repeats. The second extracellular part is a serine – glycine rich domain (residues 641 – 1590) containing nine serine – glycine pairs. A sequence surrounding Ser^{998} and Ser^{1342} conforms to the consensus sequences for chondroitin sulfate attachment sites, thus, this region can be recognized as glycosaminogylcan attachment domain. The last extracellular part of NG2 core protein is a second cysteine – rich domain which consists of 634 amino acids and contains eight other cysteine residues. Six of them are clustered within a stretch of 35 amino acids near the transmembrane domain. There is a sequence of 15 amino acids within this domain (residues 2050 – 2064) which has a 40% identity with a segment near the carboxy terminus of the α – subunit of human insulin – related receptor (Nishiyama et al. 1991).

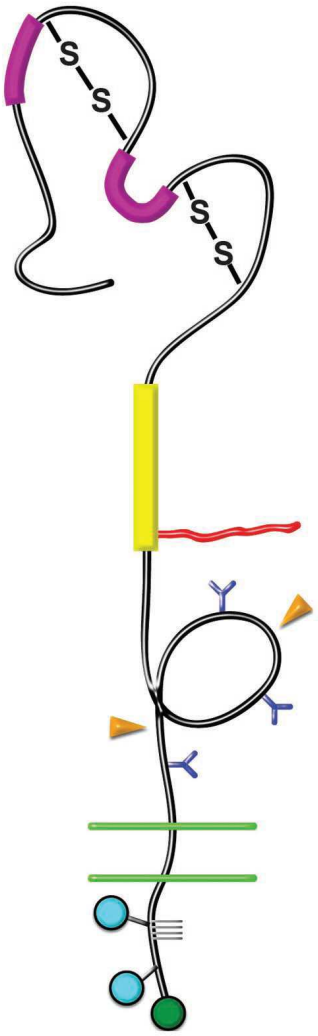

Fig. 2: **THE STRUCTURE OF NG2 PROTEOGLYCAN**

Extracellular domain:
Bold magenta bars - laminin G domains.
S-S - disulfide bonds.
Bold yellow bar - collagen binding domain.
Irregular red line - chondroitin sulfate chain.
Blue Y-shapes - N-linked oligosaccharides.
Orange arrowheads - sites of proteolytic cleavage.
Transmembrane Domain:
Double green lines - plasma membrane.
Cytoplasmic Domain:
Blue circles - sites of threonine phosphorylation.
Green circle - PDZ binding motif;
Grey grid lines - proline-rich segment.

Adapted from Stallcup, Huang: A role for the NG2 proteoglycan in glioma progression (2008)

In the extracellular part of NG2 core protein four 200 amino acid internal repeats are localized (501 – 700, 971 – 1170, 1431 – 1630, 1781 – 1980), which possess certain homology with a Ca^{2+} binding domains of N – cadherins. It is assumed that these repeats have the same Ca^{2+} binding function in NG2 proteoglycan, and more, that the Ca^{2+} stabilizes the whole NG2 structure – when EDTA is added, it causes the release of NG2 extracellular part from the cell surface. Although it is not quite clear if the proteolysis of NG2 is caused by adding EDTA or if NG2 is processed before and held together only by Ca^{2+} (Nishiyama et al. 1991).

THE CYTOPLASMIC REGION OF NG2

The cytoplasmic region of NG2 consists of 76 amino acids. There are localized three Thr residues, whose surrounding amino acids sequences conform to the motif for protein kinase C phosphorylation sites. At the extreme C-terminus, the PDZ – binding motif QYWV mediates the interaction of NG2 with the multi – PDZ scaffolding proteins MUPP1 (Barritt et al. 2000), GRIP1 and syntenin – 1 (Stegmuller et al. 2003, Chatterjee et al. 2008; according to Stallcup and Huang 2008) . The NG2 sequence has only few similarities to other known proteins, indicating that NG2 is a novel integral membrane proteoglycan. (Nishiyama et al. 1991)

NG2 MEDIATED SIGNAL TRANSDUCTION AND INTERACTIONS

The cell surface proteoglycans represent a second major class of adhesion receptors on normal or transformed cells (Tillet et al. 1997). The NG2 proteoglycan, as many other extracellular glycoproteins, possess a widespread variety of signal transduction possibilities. NG2 signaling mostly results in an enhanced cell motility, cell proliferation or survival (Figure 5). Many of NG2 interactions are described as resulting in signal transduction, including interactions with β1 integrins and growth – factor receptors. NG2 interactions with α4β1 and α3β1 integrins was shown by Iida et al., who demonstrated the ability of melanoma cells to form a focal contacts in response to the stimulation of both NG2 and α4β1 integrin (Iida et al. 1995). The study has shown a specific collaborative response of these molecules to fibronectin in comparison with laminin and collagen type IV. That suggested specificity of NG2 interaction with some types of integrins associated specifically with fibronectin adhesion. It firstly revealed that there are some tyrosine kinases involved in NG2 mediated signaling because usage of two selective tyrosine kinase inhibitors, genistein and herbimycin A, totally inhibited cell spreading on the substrata coated with α4β1integrin binding fibronectin synthetic peptide CS1 – OVA and mouse NG2 antibody. The NG2 core protein has been recognized on microspikes in cultures of melanoma cells, indicating that it is properly located to participate in molecular recognition and adhesion. When anti - NG2 mouse antibodies were added, spreading of melanoma cells was inhibited. Additionally, anti - NG2 mouse antibodies have the ability to inhibit chemotactic or chemokinectic responses to fibronectin. (Iida et al. 1995)

NG2 – COLLAGEN INTERACTIONS

NG2 is also capable of interacting with many collagen types, such as collagens II, V and VI. The collagen type VI is considered to be a primary ligand for NG2 (Stallcup, Dahlin and Healy 1990). Cells isolated from sarcoma lesions migrate extensively on collagen VI substrates *in vitro*, knockdown of the proteoglycan impairs their locomotory and invasive capabilities. The forced expression of NG2 enhances the metastatic potential of syngenic tumor cells but is significantly less effective in the collagen VI knockout mice. This fact supports the idea that the NG2 – collagen VI interaction is essential for metastasis formation. The interaction between NG2 and collagen VI seems to depend mainly on the NG2 core protein, since the chondroitinase – treated NG2 binds the

collagen as well as undigested samples. Interaction of NG2 with collagen VI is effectively inhibited by decorin, which suggests that NG2 and decorin compete for the same binding site of collagen VI. Although decorin and NG2 share no sequence homology, the central extracellular domain of NG2 contains a leucine – rich region, which probably has the same function as the classic leucine – rich motif found in decorin and other related proteins. Decorin also inhibits NG2 interaction with other collagen types, but does not affect binding of NG2 to tenascin or laminin. Tenascin, laminin and other extracellular matrix molecules bind NG2 probably through different site on proteoglycan molecule, which is specific only for NG2 and not other glycoproteins. Finally, the overexpression of decorin in Chinese hamster ovary cells was found to decerease cell proliferation, in part due to the inability of decorin – sequestered transforming growth factor β to bind to its cell surface receptors. (Tillet et al. 1997, Burg, Nishiyama and Stallcup 1997)

INTEGRIN – INDEPENDENT PHENOMENA

In order to study the functional consequences of NG2/collagen interactions, a cell line GD25 was used. GD25 cells do not express the major collagen – binding β1 integrin heterodimers, which allowed Tillet et al. to study the β1 integrin independent phenomena that are mediated by binding of NG2 to collagens V and VI. The comparison of the adhesion properties of GD25 and GD25/NG2 cells revealed a certain ability of NG2 to compensate, in its function manner, lack of β1 integrin on cell surface. (Tillet et al. 2002)

INTEGRIN – INDEPENDENT CELL ADHESION

Although there was no difference between GD25 and GD25/NG2 cells in adhering to laminin and fibronectin, the expression of NG2 markedly enhances GD25 cell adhesion abilities to both collagen V and collagen VI substrates. The primary receptors for both collagens are the integrins α1β1 and α2β1. In contrast, only very low levels of adhesion are observed with either GD25 or GD25/NG2 cells on other collagens such as collagen I or collagen IV, emphasizing the specifity of the NG2 – mediated cell adhesion on collagens V and VI. Nevertheless, expression of the β1A integrin subunit in GD25 cells restores adhesion to laminin – 1, collagen V and VI. (Tillet et al. 2002)

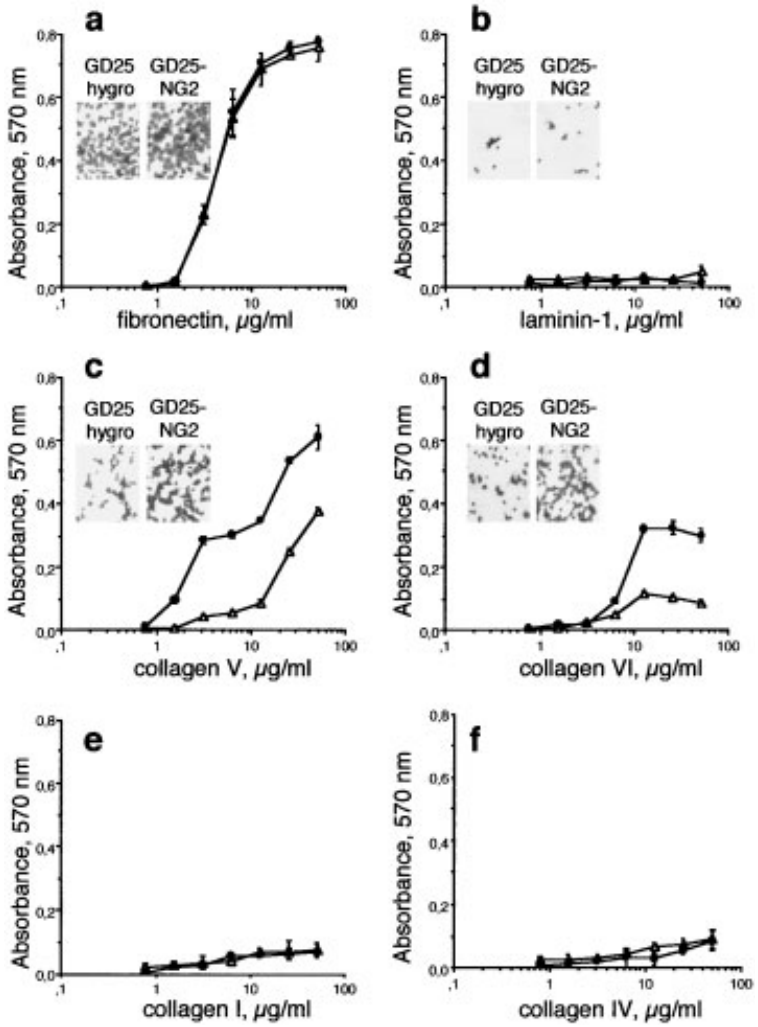

Fig. 3: **The attachment of GD25 transfectants to ECM proteins.**

GD25 cells (open triangles) or GD25/NG2 cells (*) were tested for their ability to adhere to wells coated with different ECM proteins. The insets show the stained cells prior to determination of the absorbance (for a substrate concentration of 20 mg/ml). GD25 and GD25/NG2 cells adhere equally well to fibronectin, but neither line attaches to laminin-1, collagen I, or IV. Expression of NG2 improves cell attachment to both collagen V and VI.

Adapted from Tillet et al.: NG2 proteoglycan mediates β1 integrin – independent cell adhesion and spreading on collagen VI (2002)

There is no significant difference between GD25 and GD25/NG2 cells in spreading on poly – L – lysine and fibronectin (Tillet et al. 2002). Quite surprisingly, both types of cells fail to spread on collagen V, although NG2 positive cells adhere much more efficiently to this substrate. A striking difference is observed between GD25 and GD25/NG2 cells on collagen VI. NG2 negative cells exhibit minimal spreading on collagen VI, extending only fine actin – containing protrusions on this substratum (figure 4). In contrast, GD25/NG2 cells on collagen VI are able to develop a fully spread morphology with extensively ruffled actin – positive lamellipodia. This formation is highly reminiscent of the lamellipodia formed by glioma cells plated on some types of NG2 mouse antibodies (Fang et al. 1999). Interestingly, it was not possible to observe this NG2 – mediated enhancement of cell spreading in β1A integrin positive cells. Indeed, GD25 - β1A and GD25 - β1A/NG2 cells are indistinguishable in their abilities to spread and extend lamellipodia on collagen VI substrate (Tillet et al. 2002).

Since lamellipodia are thought to form in response to activation of the small GTPase rac (Ridley et al. 1992) it seems likely that the NG2/collagen VI interaction triggers activation of rac signaling pathway. This idea is supported by finding of rac activation in glioma cells in response to engagement of NG2 by monoclonal antibody – coated surfaces (Majumdar, Vuori and Stallcup 2003).

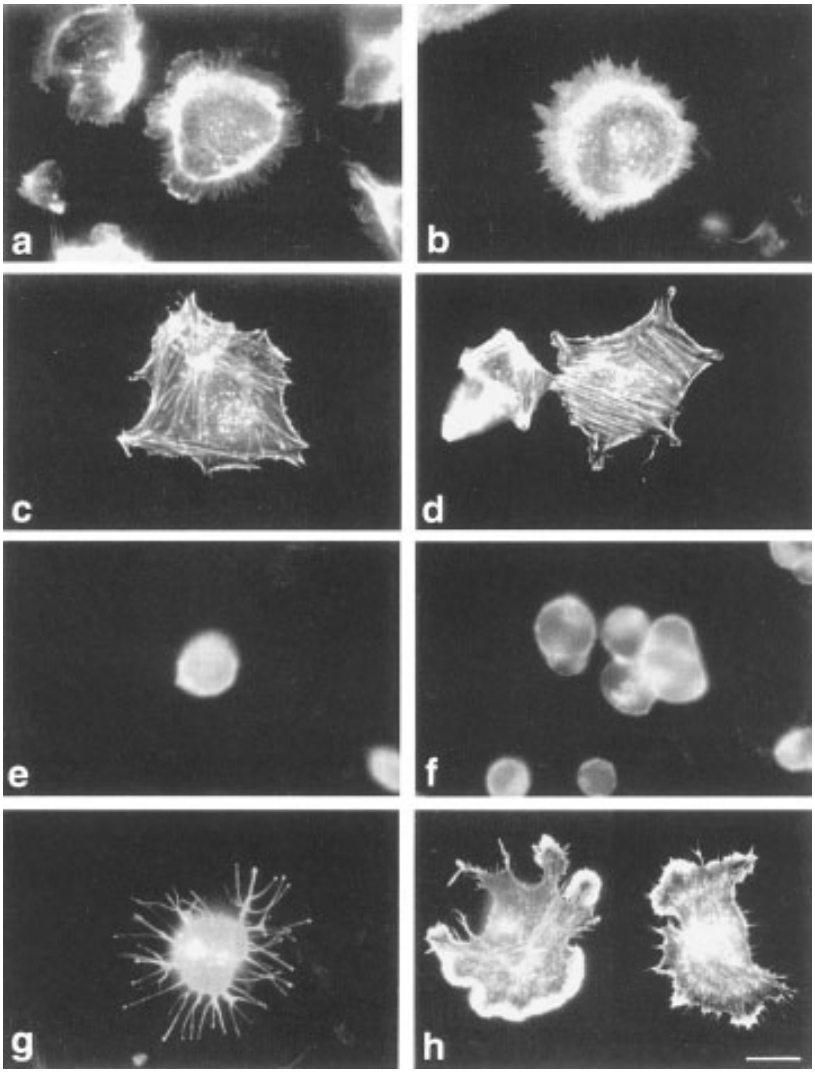

Fig. 4: **Spreading of GD25/NG2 cells on ECM proteins**.

Glass coverslips were coated with polylysine (a and b) or 20 mg/ml of the following ECM proteins: fibronectin (c and d), collagen V (e and f) or collagen VI (g and h). The adhesion of GD25 cells (a, c, e, and g) or GD25/NG2 cells (b, d, f, and h) was monitored by staining with rhodamine–phalloidin. Bar =20 mm.
Both NG2-positive and -negative GD25 cells will spread on PLL and fibronectin, while only the GD25/NG2 cells spread effectively on collagen VI. Neither cell type spreads on collagen V.

Adapted from Tillet et al.: NG2 proteoglycan mediates β1 integrin – independent cell adhesion and spreading on collagen VI (2002)

NG2 present in medium (not on cell surface) can trigger endothelial cell motility and the formation of multicellular networks (Fukushi, Makagiansar and Stallcup 2004). The study revealed an ability of mouse aortic endothelial cells (MAECs) to migrate through transwells covered with NG2/EC. This migration was almost completely inhibited in the presence of rabbit polyclonal anti - NG2 antibody, suggesting that this migration depends on the presence of NG2. Additionally, MAECs are able to adhere on collagen I coated surfaces, which compared to data showed by Tillet et al. shows a probable role of integrins (which are expressed in MAECs) in process of adhering to type I collagen coated surfaces (Tillet et al. 2002).

Galectin – 3 was identified as an endothelial receptor for extracellular NG2 (Fukushi et al. 2004). However, the nonmembrane spanning character of Galectin – 3 does not suggest a direct NG2-mediated signaling mechanism. Since both NG2 and Galectin – 3 are able to bind β1 integrins, the identification of α3β1 integrin as mediator of NG2 signaling was quite predictable. It was shown that NG2, Galectin – 3 and α3β1 integrin form a complex on the cell surface, and Galectin – 3 dependent oligomerization may potentiate NG2 – mediated activation of α3β1 integrin . In addition, the coexpression of NG2, α3β1 integrin and Galectin – 3 by some type of neoplasms suggests that investigation of this complex may yield valuable information about the behavior of tumor cells (Fukushi et al. 2004).

DIFFERENTIAL PHOSPHORYLATION OF NG2

One of the most important NG2 mediated mechanisms through which the cell behavior seems to be regulated is the differential phosphorylation of two threonine residues in NG2 extracellular domain, in concrete Thr^{2256} and Thr^{2314}. (Makagiansar et al. 2007)

Thr^{2256} and Thr^{2314} are crucial for the NG2 – mediated regulation of the balance between cell proliferation and motility. The Thr^{2256} phosphorylation is mediated by protein kinase Cα (PKCα) and stimulates cell motility and Thr^{2314} phosphorylation is promoted by extracellular signal – regulated kinase (ERK) resulting in enhanced cell proliferation. The cause of different effect of both phosphorylations seem to be dependent on β1 integrin activation (mentioned above), specially the resulting site of NG2 – integrin complex localization (Makagiansar et al. 2007).

The distinct effect of the NG2 differential phosphorylation on cell motility was shown in comparison of various NG2 transfected U251 cell lines. The NG2 - T2256E transfectants exhibit a strongly enhanced migration even under basal conditions (no PDGF – BB). The glutamic acid substitution at Thr^{2314} does not have any significant effect on the basal cell motility. Although PDGF – BB has a small stimulatory effect on parental U251 cells via NG2 – independent mechanisms, the expression of NG2 permits an additional increase in the PDGF – BB induced motility via the PKCα mediated phosphorylation at Thr^{2256}. For T2256V transfectants, a significant loss of responsiveness was reported. This is explained by the inability of PKCα to phosphorylate the mutated site. It is important to note that the T2314V mutation does not exhibit the same loss of responsiveness as T2256V transfectants. This supports the idea of different role of both threonine residues in the cell response induction. (Makagiansar et al. 2007)

Because of the reported interactions between NG2 and β1 integrins and the known ability of exogenous NG2 to activate α3β1 integrin signaling on the endothelial cell surface, the cell motility studies were repeated in the presence of β1 integrin blocking antibody P4C10, which inhibits both the PDGF – BB enhanced motility of U251/NG2 cells and the spontaneous motility of NG2 - T2256E transfectants. These results suggest that the enhanced motility of U251/NG2 cells in response to PDGF – BB is a β1 integrin dependent process which is at least partially dependent on the Thr^{2256} phosphorylation. (Makagiansar et al. 2007)

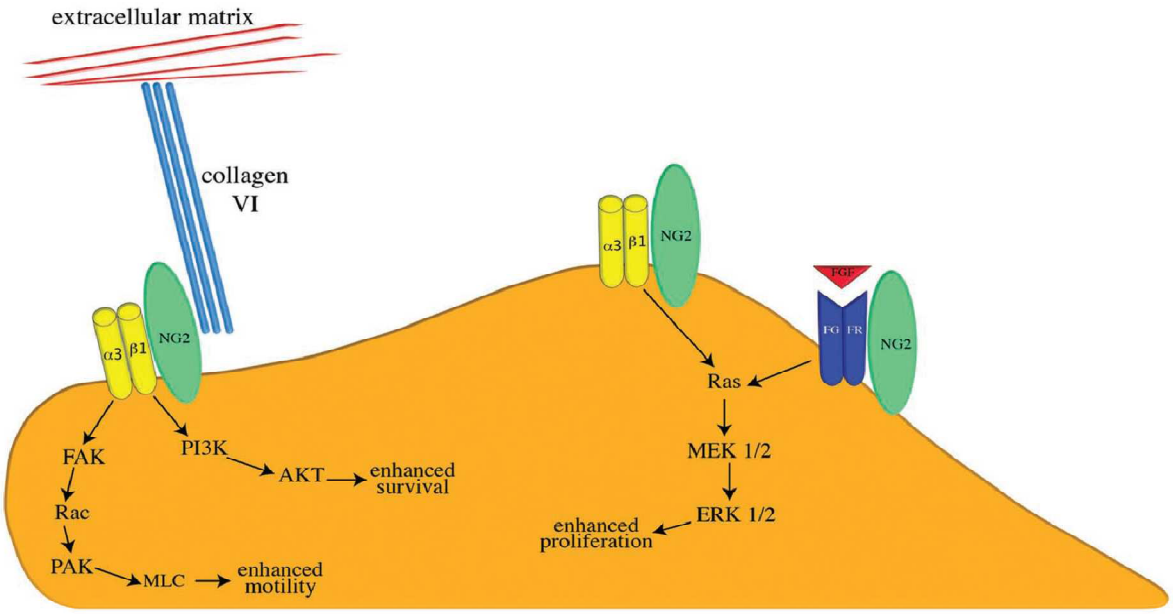

Fig. 5: **FUNCTIONAL INTERACTIONS OF NG2**.

On the apical cell surface, the Thr^{2314} phosphorylated NG2 proteoglycan activates α3β1 integrin signaling to promote enhanced proliferation. NG2 also promotes cell proliferation via potentiation of the growth factor/growth factor receptor signaling. On the leading edge lamellipodia, the Thr^{2256} phosphorylated NG2 activates the α3β1 integrin signaling to promote enhanced motility. The NG2-mediated integrin signaling can also enhance cell survival via the PI3/AKT pathway. NG2 also provides a linkage between the cell surface and the extracellular matrix via its interaction with the type VI collagen.

Adapted from Stallcup, Huang: A role for the NG2 proteoglycan in glioma progression (2008)

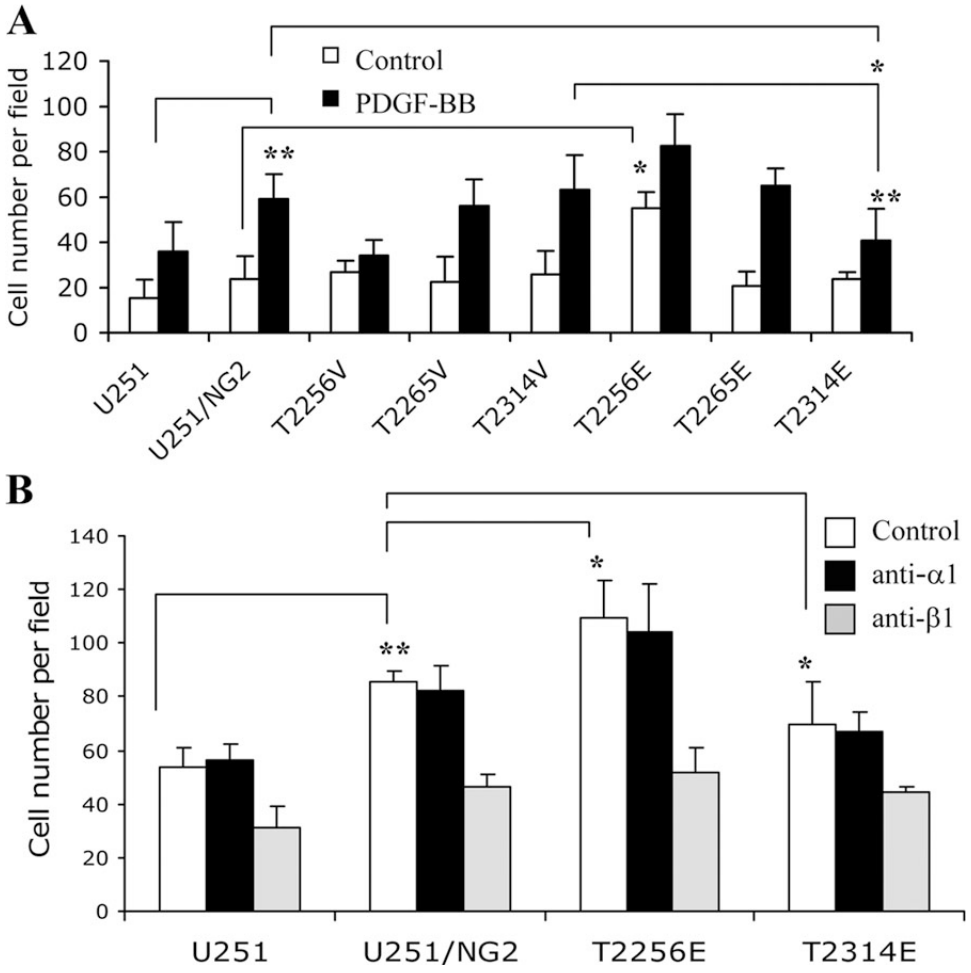

Fig. 6: **Phosphorylations of NG2 at Thr2314 and Thr2256 have opposing effect on cell motility**

(A) Several U251/NG2 variants were assayed in transwell chambers for basal (open bars) and PDGF-BB – induced (closed bars) motility. The NG2 expression causes the enhancement of the basal effect of PDGF-BB seen in parental U251 cells. The stimulatory effect of NG2 phosphorylation at Thr^{2256} is seen in the enhanced basal motility of the NG2 - T2256E variant and in the inability of the NG2 - T2256V variant to respond to PDGF-BB. An inhibitory effect of the Thr^{2314} phosphorylation is seen in the reduced PDGF-BB response of the NG2 - T2314E variant.

(B) Effects of integrin-blocking antibodies on the PDGF-BB – induced motility of various U251 transfectants. Anti – β1 integrin antibody reduces the increased motility that results from the actual or mimicked phosphorylation of NG2 at Thr^{2256}. The control anti – α1 integrin antibody has no effect on motility.

Adapted from Makagiansar et al.: Differential phosphorylation of NG2 proteoglycan by ERK and PKC alpha helps balance cell proliferation and migration (2007)

The interaction of NG2 with type VI collagen, which stimulates cell motility, suggests that NG2/matrix interactions can result in activation of the cytoskeletal machinery required for cell migration. This example implies that NG2 might be able to interact, in some way, with actin cytoskeleton. In cells that are well spread on the substratum, NG2 is arranged on the cell surface in linear arrays that are codistributed with actin and myosin containing stress fibers in the cytoplasm (Barritt et al. 2000). In migrating cells, NG2 is associated with actin – containing retraction fibers on the trailing edge of the cell (Lin et al. 1996)

MUPP1, multi – PDZ – domain scaffold protein, contains thirteen PDZ domains. These PDZ modules can interact with specific carboxy – terminal motifs present in a variety of transmembrane and intracellular proteins, including NG2. The importance of the C – terminal portion of the NG2 cytoplasmic domain for interaction with MUPP1 is illustrated by the failure of the truncated NG2/t3 mutant to yield positive results in pull – down assays using fusion proteins representing the first MUPP1 PDZ domain. (Ullmer et al. 1998, Barritt et al. 2000)

The NG2 binding capability of all 13 MUPP1 PDZ domains remains to be assessed in detail. The PDZ pairs 10/11 and 12/13 failed to bind NG2 in the pull – down assay, the 2/3 pair has activity comparable to that of PDZ – 1. There is no evidence whether PDZ – 1 and PDZ – 2/3 interact with the same segment of NG2 C – terminus but there appears to be a significant preference of NG2 for binding to PDZ domains in the N – terminal portion of MUPP1. Other known MUPP1 ligands such as the 5-HT_{2C} receptor (Ullmer et al. 1998) and the viral 9ORF1 protein appear to bind to PDZ domains in the C – terminal part of MUPP1. This gives this scaffolding protein selectivity in its ligand binding (Barritt et al. 2000).

Since PDZ – containing molecules are postulated to be involved, among others, in localization of cell adhesion molecules, binding of NG2 to MUPP1 could provide a mean for linking NG2 to other molecules that have a cytoskeletal localization. Engagement of NG2 by the substratum induces reorganization of the actin cytoskeleton leading to changes in cell morphology and cell motility (Fang et al. 1999). This is consistent with the ability of NG2 to activate intracellular signaling cascades that control cytoskeletal

dynamics (Monnier et al. 2003). Binding of NG2 to MUPP1 could provide a link to these signaling pathways.

POSSIBLE INFLUENCE OF NG2 TO RHO-A GTPASE

A controlled regulation of RhoA GTPase activity is essential for mediating the growth – factor stimulated migration (Ernkvist et al. 2009). In cancer cells, the influence of Rho/ROCK cascade could play a significant role in promoting various processes, including migration.

The scaffolding protein MUPP1, which was described earlier in this work, is able to participate in controlling of RhoA GTPase activity in migrating endothelial cells (Ernkvist et al. 2009). A G – nucleotide exchange factor (GEF) Syx is in endothelial cells targeted to plasma membrane and stimulates migration (Liu et al. 2003, Ernkvist et al. 2009). Syx also contains a PDZ – binding domain, which makes it capable of binding a PDZ – containing molecules (Ernkvist et al. 2009), including MUPP1 or its paralogue Patj (Pals – associated tight junction protein). MUPP1 and Patj are two mammalian homologues of the *Drosophila* Dpatj. Both of the proteins contain multiple PDZ domains (13 and 10, respectively). These domains have capabilities to bind some identical proteins. Both MUPP1 and Patj serve as scaffolding proteins that help to organize higher – order protein complexes. (Ernkvist et al. 2009)

The mass spectrometry analysis revealed that the PDZ – binding domain of Syx associates with MUPP1, Pals1 and Lin7. The cytoskeletal proteins myosin 1c, tubulin and actin were also coimmunoprecipitated with the Syx – binding domain (Figure 8) (Ernkvist et al. 2009).

An interaction between Syx and Patj was also observed. The data showed that binding could occur through PDZ – 3 domain of Patj, but for interaction of Syx with Patj is PDZ – 10 of Patj the major binding domain (Ernkvist et al. 2009). Since the NG2 proteoglycan binds the Patj paralogue MUPP1 through its PDZ – 1 or PDZ – 2/3 domain, it seems quite possible that MUPP1 can mediate the signaling from NG2 to Syx, or even from NG2 to RhoA. It could be hypothesized that NG2 could stimulate cell motility through RhoA signaling.

This hypothesis can be supported by the facts mentioned earlier, that NG2 in complex with other CSPGs in media has an inhibitory effect on neuron outgrowth, and that inhibition of Rho kinase (ROCK) increases neurite outgrowth *in vitro* (Monnier et al. 2003, Lingor et al. 2007), which postulated a connection between CSPGs (NG2 included) in media and Rho/ROCK cascade. The MUPP1 – Syx – RhoA connection could be the searched pathway for signal transduction between NG2 and RhoA or actin cytoskeleton respectively.

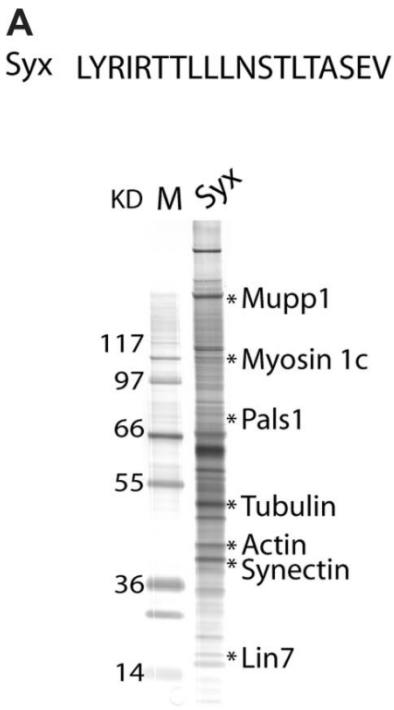

Fig. 8: **Identification of proteins associated with the PDZ – BINDING motif of Syx.**

Adapted from Ernkvist et al.: The Amot/Patj/Syx signaling complex spatially controls RhoA GTPase activity in migrating endothelial cells (2009)

CONCLUSIONS

Although the NG2 proteoglycan was discovered almost thirty years ago – and its functions, as it was studied in normal cells, are known quite well – the role of the NG2 proteoglycan in cancer is not very clear. This work is focused only on some of the aspects, for which cancer is nowadays known as a feared, lethal disease. These aspects are uncontrolled tissue growth, high robustness of cancer cells and finally the ability of cancer cells to migrate through the ECM and to create metastase.

This work shows some of the possible ways in which the NG2 proteoglycan is capable of affecting cancer cell motility. The transmembrane location makes it an ideal signaling molecule; the high variability of its extracellular part implies lot of potential interactors. It can also act as an adhesion molecule, substituting or supplementing the classic cell adhesion proteins such as integrins in interactions with various substrates, like collagen types II, V and VI. The primary ligand for NG2 is considered to be the type VI collagen.

The key role of differential phosphorylation is shown, when the phosphorylation of Thr^{2256} of NG2 by protein kinase Cα drives the cell into activation of actin cytoskeleton and increased motility, while the phosphorylation of Thr^{2314} by extracellular – regulated kinase has a major effect in increased proliferation.

The interaction of NG2 with multi – PDZ – domain protein MUPP1 is mediated mainly with PDZ – 1 domain of MUPP1, although PDZ – 2/3 domain can be involved as well. Since MUPP1 paralogue Patj can bind the guanosine exchange factor Syx with its PDZ – 10 domain, this work suggested a possible signaling pathway NG2 – MUPP1 – Syx – RhoA GTPase. This pathway could be the molecular explanation for earlier observations, when NG2 in complex with other CSPGs in media inhibited axon outgrowth of neural cells and this inhibitory effect was compensated by the selective inhibition of Rho/ROCK cascade. The potential selective inhibition of this cascade could result in a significant weakening of invasive abilities of cancer cells.

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