UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Fisiopatologia Sistemica

Ciclo XX

ROLE OF GLYPICAN-6 AND NG2 AS METASTASIS PROMOTING FACTORS

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To Indy

L'anima libera e' rara, ma quando la vedi la riconosci: soprattutto perché provi un senso di benessere, quando gli sei vicino.

(Charles Bukowski)

Summary

Cell surface proteoglycans (PGs) are key molecules in the regulation of tumour progression and metastasis formation. Eleven primary surface PGs, including syndecans-1-4, glypicans-1-6 and NG2, are currently known to act as mediators of the cancer cell's interaction with the host microenvironment, with the potential to function synergically or antagonistically in the promotion of tumour growth and spreading. Using soft-tissue sarcomas as a model system we have observed that a given tumour cell may constitutively and coincidently express on average 3-5 of the 11 surface PGs, suggesting that diverse combinations of surface PGs may dictate the behaviour of cancer cells in different manners. To start to investigate the PG surface profiles as pro- and anti-tumorigenic we delineated strategies to modify the PG expression of 143B osteosarcoma cells by stable gene transduction and by examining how these modifications affected the cells adhesive and migratory capabilities in response to selected ECM substrates, and endothelial monolayers. To date there are no notice about GPC6 implications either in cell-ECM interactions or in the behaviour motility of cells. For these reasons 143B cells were stable transfected for overexpressing GPC6 showed a modulation of relative expression of all surface PGs respect to vector control cells. GPC6 overexpression induces morphological modification in 143B cells which are seen as changes in the organization of actin filaments containing protrusions similar to fillopodia/lamellipodia and an increase of motility cells in monodimensional assay which could be in relationship with cytoskelatal reorganization. Moreover these cells showed a spreading ability and the lower invasion ability on different ECM molecules that could be correlated by surface PG profile modulation. Since NG2 as a cell surface ligand for collagen type VI has been postulated to be involved in tumor progression, we have also examinated the interaction of the NG2+ and NG2- sarcoma cells with collagen type VI and other ECM molecules. Sarcoma cells were NG2 abrogated by RNAi or cells immunosorted for NG2 expression were found to exhibit a rather elective, impaired ability to adhere and migrate on purified collagen type VI. These findings confirmed that the NG2 was capable of mediating tumour cell adhesion and migration through interaction with this specific collagen. The outcome of these investigations provide a first evidence that NG2 may represent a unique, malignancy promoting factor in several types of soft-tissue sarcomas and that defined surface PGs pattern differentially control tumour progression, with some profiles being specifically associated with an aggressive behaviour, whereas others with a more benign phenotype.

1. Introduction

1.1. Proteoglycans (PGs)

Proteoglycans (PGs) are glycoproteins that can be found on the cell surface and extracellular matrix (ECM) where they mediate critical interactions between cells and their environment. They consist of a core protein with one or more covalently attached glycosaminoglycan (GAG) chain(s). PGs are classified both on the basis of their localization and on the type of the core protein and GAG-chain composition (**Fig. 1**).

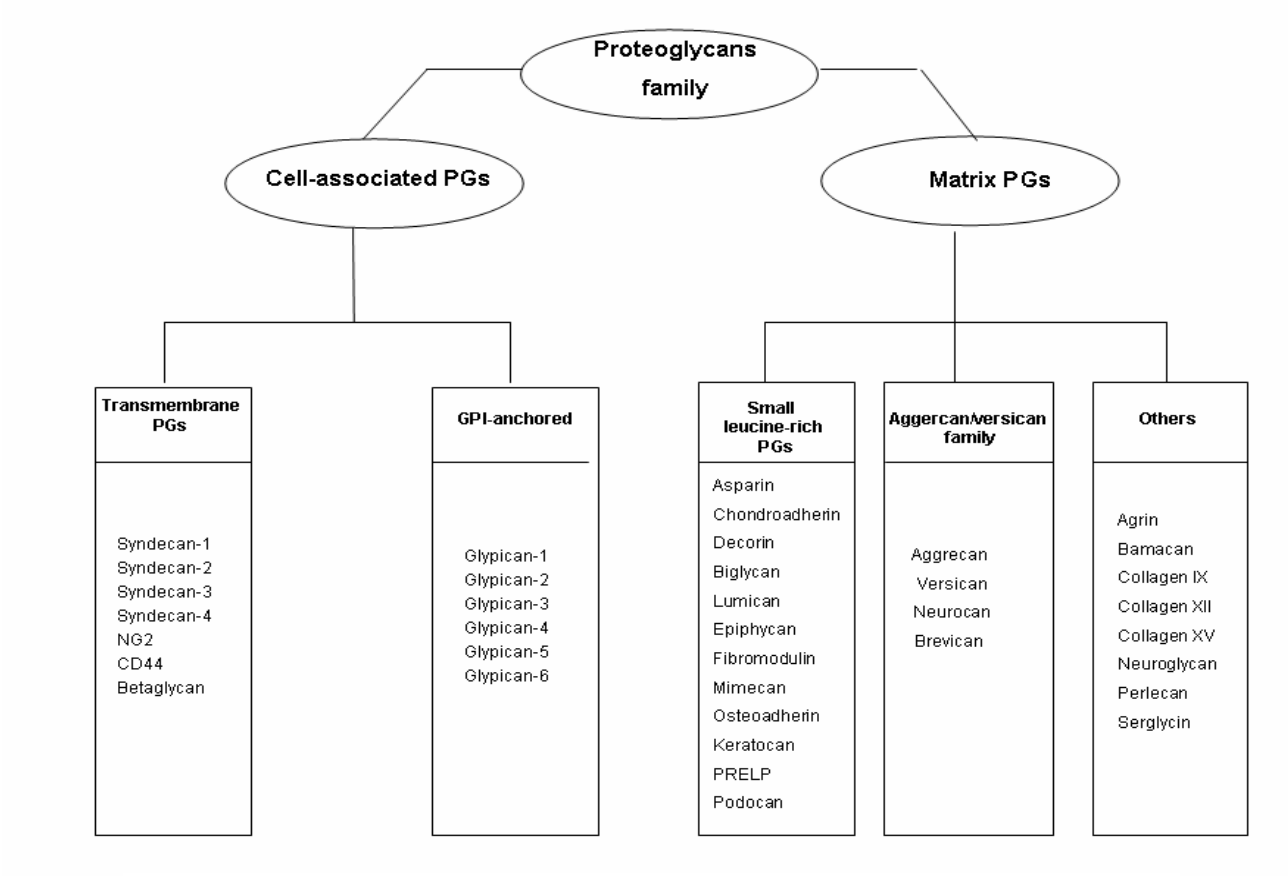


Figure 1. Simplified diagram showing the major PG families.

Heparan sulphate (HS) and chondroitin sulphate (CS) chains are bound to membrane associated PGs such as glypicans, syndecans and NG2. PGs found in the extracellular matrix (ECM) may also carry keratin (KS) and dermatan sulphate (DS) GAG chains.

Next to collagens, PGs constitute a major class of extracellular matrix (ECM)/cell surface components known to be involved in both primary physiological and pathological phenomena. In vertebrates, PGs are among the first ECM constituents to be produced during embryonic development and are often aberrantly expressed in a variety of inherited and acquired disorders. Surface expression of certain PGs also appears to be an early embryonic event and, paradoxically, due to the altered transcription/translation patterns that these PGs exhibit, they have been identified as potential diagnostic/prognostic and therapeutic targets in diverse disease states. Several cell surface-associated PGs, and at least one ECM PG, are widely expressed throughout embryonic and adult life of invertebrates, underscoring the highly evolutionary conserved nature of these

macromolecules and their multivalent biological role. Based upon its direct involvement in cell–cell and cell–ECM interactions, this gene family has been strongly implicated in the regulation of cell movement (**Table 1**)

Table 1. Predicted functional traits of PGs involved in the regulation of cell movement[1] (From Cattaruzza and Perris, 2005)

PROTEOGLYCAN	Haptotactic ECM component[2]	Non permissive directional cue[3]	ECM linker of direction-promoting cues[4]	Shedded motility-promoting factor[5]	Cryptic motility-promoting factor[6]	Shedded motility-inhibitor[7]	Cell surface interactor[8]	Enhancer of signal transduction[9]	Cell-ECM co-receptor[10]
Versican V0-V2	X				(X)		X		
Versican V3		X						(X)	
Aggrecan		X	X						
Neurocan		X	X						
Brevican	(X)	X	X						
CD44						(X)	X	X	X
Perlecan		X	X		X				
Decorin		X	X						
Biglycan		X							
Fibromodulin		X							
Keratokan		X							
Lumican	(X)	X							
NG2						(X)	X	X	X
Syndecan-1						X	X	X	X
Syndecan-2						X	X	X	X
Syndecan-3						(X)		(X)	X
Syndecan-4						(X)	X	X	X
Glypican-1							X		
Glypican-2		X	X				X		X
Glypican-3						X	X		
TENB2							X		
Neuroglycan-C		X							
Phosphocan		X					X		X

[1]Based upon both published and unpublished observations;
[2]Acting alone or in combination with other ECM components as a haptotactic motility factor;
[3]Regulator of directionality of cell migration by acting as a non-permissive ECM substrate component;
[4]Capable of sequestering chemotactic molecules and cell growth- and motility-promoting factors in the ECM;
[5]Enhancer of cell motility when shedded from the cell surface;
[6]Promoter of cell motility following proteolysis;
[7]Acting as motility-inhibiting factor when shedded from the cell surface;
[8]Engaded in multivalent interactions with cell surface components directly or indirectly involved in the control of cell motility;
[9]Directly mediating signal transduction cascades involved in the regulation of cell movement;
[10]Specifically serving as co-receptor for ECM components and thereby implicated in the regulation of cell movement.

However, how PGs actually affect this process is only partially understood and in some instances controversial. It is currently believed that both normal and tumour cells may

adapt their modality of locomotion according to the environment through which they migrate and by adjusting their migratory strategy from ‘‘path generating’’ to ‘‘path finding’’. To accomplish this, cells co-ordinately rearrange the surface organization of integrins, PGs, cell adhesion molecules, and membrane-bound metalloproteinases (Wolf et al., 2003). Thus, intricate patterns of cooperation between PGs and other cell surface components may be envisioned and the dissection of these interplays is fundamental to understand how cell movement is regulated

1.2. Membrane-associated proteoglycans

In the last year cell surface PGs have been at the centre of numerous studies because of their multifunctional nature. Some PG core proteins have transmembrane and cytoplasmatic domains, such as syndecans and NG2 forming direct molecular linkages between the extracellular and intracellular environments. Whereas glypicans are linked to the plasma membrane with glycosylphosphatidylinositol (GPI)-anchor, suggesting that these molecules may function in concert with changes in phospholipids metabolism and signalling (**Fig. 2**).

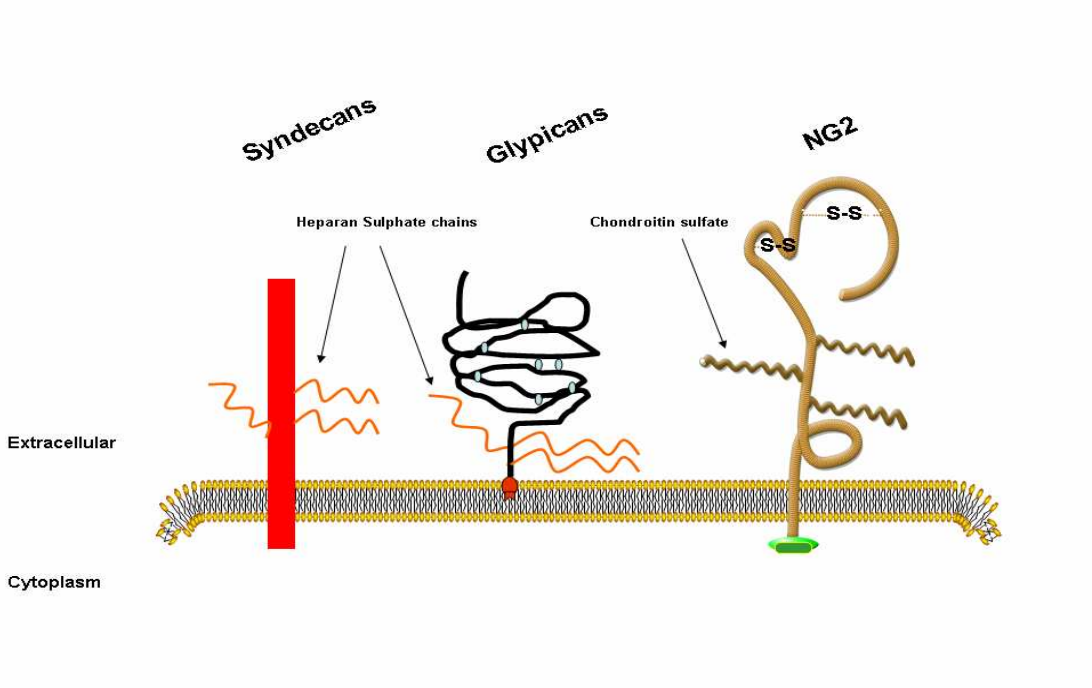


Figure 2. Three main classes of cell-surface PG. Syndecans and glypicans structure with HS chains. Syndecans can also contain a chondroitin sulphate (CS) chain(s). While NG2 proteoglycan can contain only CS chains.

Glypicans and syndecans are heparan sulphate proteoglycans (HSPGs) and their core proteins (40-70KDa) are linked to HS GAG chains characterized by alternating of *N*-acetyl glucosamine (GlcNAc) and glucuronic (GlcA) or iduronic acid residues (Bernfield et al., 1999). These HS chains are responsible for much of the HSPGs biological roles and the core proteins have evolved to maximize their efficiency in these roles. NG2 with a core

protein of 550 and a size of the full molecule of 600KDa is a unique chondroitin sulphate transmembrane proteoglycan, characterized of alternating sugars (N-acetylgalactosamine (GalNAc) and glucuronic acid. Unlike to HSPGs, NG2 PGs core protein can be expressed on the cell surface either with or without chondroitin sulphate GAG chains, placing NG2 in the category of part-time proteoglycans (Stallcup et al., 1990). Membrane PGs regulate the distribution of extracellular signalling molecules such as morphogens and chemokines, and modulate signalling events at the cell surface that influence cell fate determination, proliferation, adhesion and motility. They participate in endocytosis and vesicular trafficking, regulating the movement of molecules between intracellular and extracellular compartments. Several of them have assumed important roles as prognostic factors and a few of them are now contemplated for therapeutic applications. Literature data and our preliminary observations indicate that given tumour cells may constitutively and coincidently express on average 3-5 surface PGs, suggesting that given combinations of surface PGs may diversely dictate the behaviour of cancer cells, during distinct phases of tumour progression.

1.3. Syndecans

Syndecans are transmembrane heparan sulphate and comprise a family of four distinct genes: SDC1, 2, 3 and 4. Their chromosomal locations, exon organization, and sequence relationships with the single *Drosophila* syndecan (D syndecan) suggest that the gene family arose by gene duplication and divergent evolution from a single ancestral gene, and that SDC-1 and SDC3 and SDC2 and SDC4 represent subfamilies (Bernfield et al.1999; **Fig. 3**). Each gene product is a single type I membrane-spanning protein with an apparently extended extracellular domain of variable size that contains covalently attached HS chains distal from the plasma membrane.

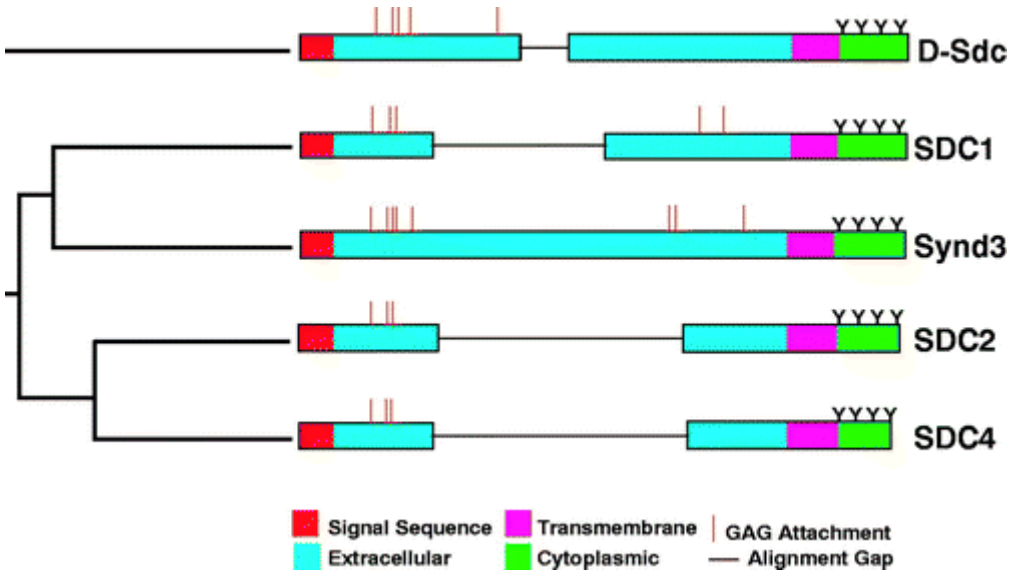


Figure 3. Dendrogram suggests that the *D-Sdc* gene is derived from a common ancestor of the four mammalian syndecan genes and that SDC1 and Synd3 form one subfamily and SDC2 and SDC4 form another. (Modified from Spring et. ,1994)

Syndecan core proteins (40-50 KDa) contain three different functional domains: the extracellular, cytoplasmatic and transmembrane domain. The extracellular domain (ectodomain) is among the most rapidly diverging vertebrate proteins with the exception of their regions for GAG attachment, cell interaction, proteolytic cleavage, and oligomerization. Ectodomain contains two regions for GAGs attachment (Esko et al., 1996; Bourdon et al., 1989). SDC1, 3 and 4 also contain Ser-Gly sequence near the plasma membrane that may serve as attachment sites for CS (Kokenyesi et al., 1994). Transmembrane domain is evolutionarily relatively stable and only a few amino acids differ among the vertebrate sequences. It contains the regions for interactions with other membrane proteins and for localization to distinct membrane compartments. Transmembrane domain may also interact within the plane of the membrane with proteins involved in cell spreading, sensitive to protein tyrosine kinase inhibitors (Lebakken et al, 1996). Despite of the molecular volume of their HS chain it is possible to find these PGs in oligomers form, especially in dimmers. Both syndecans bearing small HS chains (i.e. produced by keratinocytes) and syndecans devoid of HS chains are inclined to form oligomers Sanderson et al., 1992). Oligomerization would enhance the proximity between syndecan core proteins, enlarging their interaction surface, and increasing the probability of interaction with other membrane proteins (Klemm et al, 1998). The cytplasmatic domains contain two invariant regions, a membrane proximal common region (C1) containing a serine and a tyrosine and a C-terminal common region (C2), separated by a region (V) of variable length and composition. The C2 region shows an EFYA sequence at the C-terminus that can bind to the PDZ domains present in intracellular proteins. PDZ

domains organize and assemble protein complexes on the inner surface of the plasma membrane and are thought to link membrane components to the underlying actin-containing cytoskeleton. The variable (V) region is distinct for each of the 4 family members, suggesting functional difference between these PGs (Bernfield et al., 1999). The function of this domain is largely unknown except for SDC4, where it is responsible for the assembly of SDC4 tetramers with phosphatidylinositol 4,5-bisphosphate (PIP2) and activated protein kinase C-α (PKC-α) in focal adhesions (Woods et al, 2001; Rapraeger et al., 1998). Instead SDC2 V region serve only as substrata for PCKα (Itano et al., 1996) Syndecans can be involved in growth control, cell spreading, cellular recognition, cellular adhesion, and signalling and. Their function as co-receptors with integrins and cell-cell adhesion molecules (fibronectin, vitronectin, laminins, and the fibrillar collagens; Bernfield et al, 1999; Thodeti et al., 2003). All syndecans studied can be shed from the cell surface by proteolytic cleavage near the plasma membrane (Fitzgerald et al., 2000). Shedding of the syndecan ectodomains is highly regulated and can be accelerated by various cellular effectors (thrombin and plasmin), which, interestingly, do not usually bind HS. Syndecans expression levels are regulated during development in morphogenesis, cell differentiation and they can be altered in tumorigenesis, progression and metastasis process (Sanderson et al., 2001).

1.4. Glypicans

The first member of the family of GPI-anchored heparan sulphate (HS)-substituted PGs, later named glypicans (GPC), was identified some 15 years ago (David G. et al., 1990). Glypicans comprise a family of at least six distinct genes in mammals GPC-1,-2,-3,-4,-5,-6 (Filmus 1998, Watanabe 1995) two in *Drosophila,* called Dally and Dally-like (Nakato et al., 1995; Baeg et al., 2001) and one *C. elegans*, called knypek (Fransson et al. 2004*)*. All glypicans have an N-terminal signal sequence; an ~60-70 kDa globular domain with a characteristic pattern of 14 highly conserved cysteine residues (CRD region), with the HS attachment sites; and finally a C-terminal sequence involved in formation of a GPI for linking to the plasma membrane. Except for the HS attachment sequences, the HS-bearing juxtamembrane regions are the most divergent sequences in glypican core proteins (Bernfield et al., 1999). The structures of each glypican are extremely well conserved across species (more than 90% identical when comparing glypicans from different vertebrate species; **Fig. 4**).

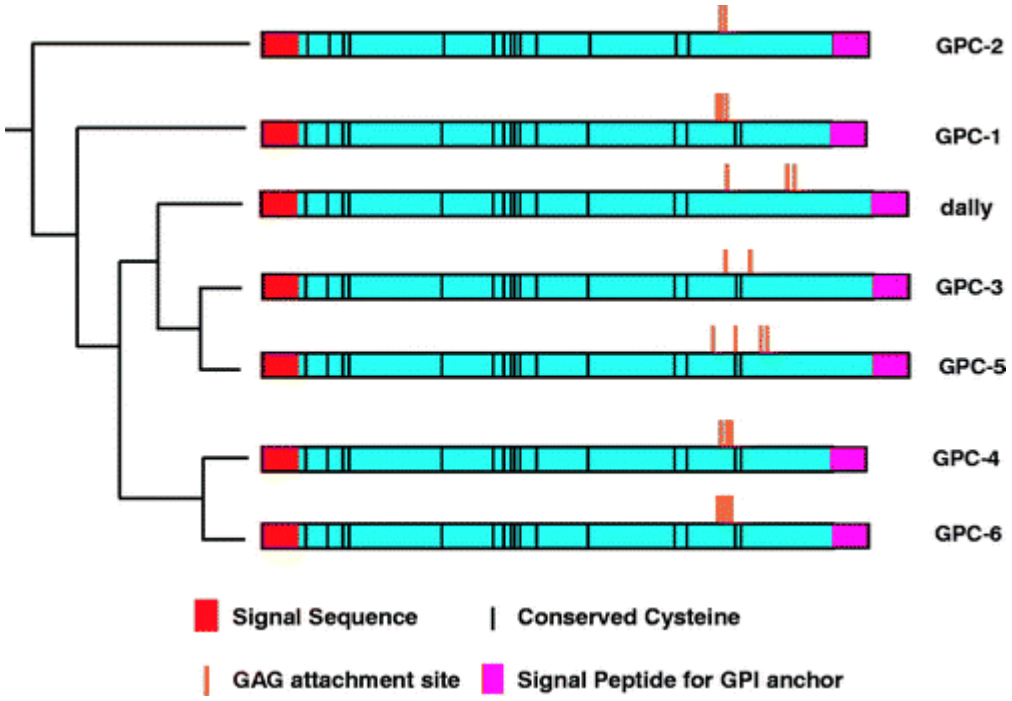


Figura 4. Diagrams showing the derived core protein domain organization, locations of putative GAG attachment sites, dendrogram and aligned sequences of human glypican-1 through -6 and *dally*, the *Drosophila* glypican homolog (from Bernfield 1999)

The hypothesis of different subfamilies is further strengthened by an analysis of the genomic structures of the corresponding genes as follows: GPC1, GPC2, GPC4, and GPC6 consist of nine exons, whereas GPC3 and GPC5 contain only eight exons (Veugelers et al., 1998; Huber et al., 1997). The GPC1, 2, 4, and 6 form a separate group with high homology (40–60%) to each other but only 20% identity to the other group comprising GPC3 and GPC5 (also 40% identical to each other). Interestingly, GPC6 is most homologous to GPC4 (both proteins are 63% identical). This suggests glypicans arose from a series of gene and genome duplications and may herald extension of the gene family by additional members and gene clusters (Bernfield et al., 1999). Whether members of the same subfamily share some common functions and members belonging to different subfamilies have different functions remains to be established. Glypicans are linked to membrane lipid without penetring the bilayer. Currently, the functional significance of attaching proteins through a GPI anchor has not been clearly established. One of these possible roles is the targeting of GPI-anchored proteins to specific micro-domains within the cell membrane called ‘rafts’ (Brown et al., 1992) that can facilitate interactions with specific intracellular signalling molecules in the absence of a cytoplasmic domain (Ilangumuran et.al., 2000). GPI anchors may also be to provide a system of regulated release of proteins to the extracellular environment with releasing from cell surfaces by proteolytic cleavage and by the action of a phosphatidyl inositol-specific phospholipase C (Ishihara et al, 1997). Moreover, the anchor is reported to mediate the

turnover of cell surface components by rapid endocytosis and transport to lysosomes (Yanagashita et al., 1998). In general, glypicans are expressed predominantly during development (De Cat et al., 2001), the expression levels have been shown to change in a stage- and tissue-specific manner suggesting that glypicans are involved in morphogenesis (Saunders et al.,1998; Pellegrini et al.,1998; De Cat et al.,2001; **Table 2**)

Table 2. The glypican family during embryogenesis and in adult tissues (adapted from Fico et al., 2007).

Name	Original Designation	Expression in Embryo	Expression in Adult	Reference
Glypican 1	Glypican	Bone, bone marrow, muscle epidermis, kidney	Most tissues	David *et al.*, 1990; Litwack *et al.*, 1994
Glypican 2	Cerebroglycan	Nervous system	Not detected	Stipp *et al.*, 1994; Ivins *et al.*, 1997
Glypican 3	OCI-5	Most tissues	Ovary, mammary gland, mesothelium, lung, kidney	Filmus *et al.*, 1998; Pellegrini *et al.*, 1998; Li *et al.*, 1997; Filmus (unpublished observations)
Glypican 4	K-glypican	Brain, kidney, lung	Most tissues	Watanabe *et al.*, 1995; Veugelers *et al.*, 1998; Siebertz *et al.*, 1999
Glypican 5	Brain, lung, liver, kidney, limb	Brain	Veugelers *et al.*, 1997; Saunders *et al.*, 1997	
Glypican 6	Many tissues, including liver and kidney	Many tissues including ovary, kidney, liver, and intestine	Paine-Saunders *et al.*, 1999; Veugelers *et al.*, 1999	

In general, glypicans interaction of with multiple proteins may be due to the fact that the length and modifications of the heparan sulphate chains are cell-type-specific (Nurcombe et al., 1993). It has also be seen that Glypicans regulate the signalling of morphogenesis through Wnt, Hedgehog (Hh), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs; Yayon et al., 1991). In fact GPC1 and GPC3 are able to sequester a variety of growth in particular they can link TGFβ, BMP and Wnt family controlling their signalling events. Consistent with a proposed role in development, the expression patterns of these glypicans often coincide with those of growth factors, such as FGFs and BMPs and their receptors (Veugelers et al., 1999).

1.5. GPC6

Till 1999 five members of the glypican family have been identified in vertebrates. Bio-informatics analyses have led to the identification of a sixth member of the human glypican gene family and of its mouse orthologue. As might have been expected from the characterization of the other members, the human and mouse forms of Gpc-6 are highly

similar in structure, with 96% identity (**Fig. 5**). Besides GPC6 is high similar to GPC4 with 63% identity.

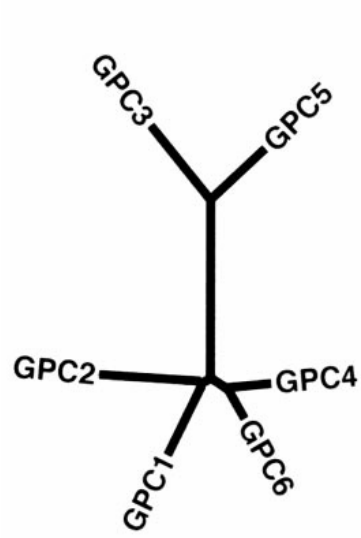


Figure 5. The lengths of the lines in this tree are directly proportional to the predicted genetic distances between individual sequences. These results indicate that glypican-4 and glypican-6 are in fact more closely related than any other pair of vertebrate glypicans previously described.

Based on similarities in sequence and gene organization, GPC1, 2, 4, and 6 appear to define a subfamily of glypicans, differing from the subfamily comprising so far GPC3 and GPC5. The GPC6 gene maps close to the GPC5 gene on human chromosome 13q32, whereas GPC4 maps to chromosome Xq26, where it flanks GPC3, the gene encoding the glypican which so far is most homologous to GPC5. Northern blotting indicates that GPC6 mRNA is widespread, with prominent expressions in human fetal kidney and adult ovary. In situ hybridization studies localize GPC6 to mesenchymal tissues in the developing mouse embryo (Sanderson at al., 1996). High expressions occur in smooth muscle cells lining the aorta and other major blood vessels and in mesenchymal cells of the intestine, kidney, lung, tooth, and gonad (Veuglers et al.,1999). There is no evidence about molecular interaction of GPC6 in cell spreading and cell invasion, so GPC6 was overexpressed in sarcoma cells for studying its involvement in adhesion and migration of cells.

1.6. NG2/CSPG4

NG2 is a chondroitin sulphate (CS) membrane-spanning protein that interacts with macromolecules on both sides of the plasma membrane. NG2 has an extensive extracellular domain of 2195 amino acids and a much smaller cytoplasmic domain of 76 amino acids. A single 25-residue transmembrane domain divides the core protein into a relatively short 76 amino acid cytoplasmic tail and an extensive 2225-residue extracellular domain (**Fig. 6**).

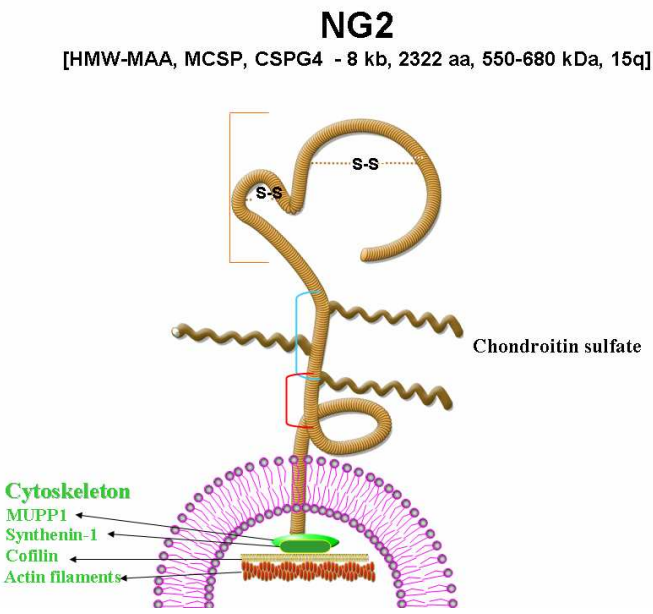


Figure 6. The N-terminal globular domain 1 is stabilized by intramolecular disulfide bonds (the actual number of disulfides is not known). The central domain 2 contains both the type VI collagen-binding site and the single chondroitin sulphate chain (irregular line). The membrane-proximal globular domain 3 contains at least two sites for proteolytic processing of NG2. The cytoplasmic tail contains a PDZ-binding motif a proline-rich segment, and several potential sites for threonine phosphorylation .

The extracellular domain of the NG2 core protein contains three subdomains: an N-terminal globular domain (domain 1), a central extended domain that has the sites for GAG attachment (domain 2), and a juxtamembrane domain (domain 3). Domain 1 has a globular conformation stabilized by intramolecular disulphide bonds (Tillet et al., 1997; Burg et al., 1997); domain 2 contains the sites for binding CS chains (Stallcup et al, 2001) and it contains α-helical site for collagen type V and VI binding (Tillet, 1997; Burg 1997); domain 3 is the globular juxtamembrane site for proteolysis of NG2 that leads to its cleavage and release from the cell surface (Stallcup et al, 2001). At the extreme C-terminus, a QYWV sequence fits the pattern for a PDZ-binding motif (Songyang *et al.*, 1997) and may be responsible for interaction of NG2 with MUPP1, a multi- PDZ domain-containing cytoplasmic scaffolding protein (Barritt *et al.*, 2000). NG2 is expressed by immature progenitor cells in several different developmental lineages, including oligodendrocyte progenitors, chondroblasts, and pericytes/smooth muscle cells (Nishiyama et al., 1991; Grako et al., 1995; 1999; Burg et al., 1999; Schlingemann et al., 1990). The expression pattern of NG2 on immature progenitor cells suggested that the NG2 might contribute to processes such as cell proliferation and motility which are critical to progenitor biology. In fact NG2 is often re-expressed by tumour cells, which are usually characterized by increased proliferation and migration.

1.7. Metastasis

Tumour cell invasion and metastasis is highly dependent on dynamic changes in the adhesion and migration of transformed and malignant cells. Tumour metastasis consists of a series of biological processes that move tumour cells from the primary neoplasm to a distant location (**Fig. 7**).

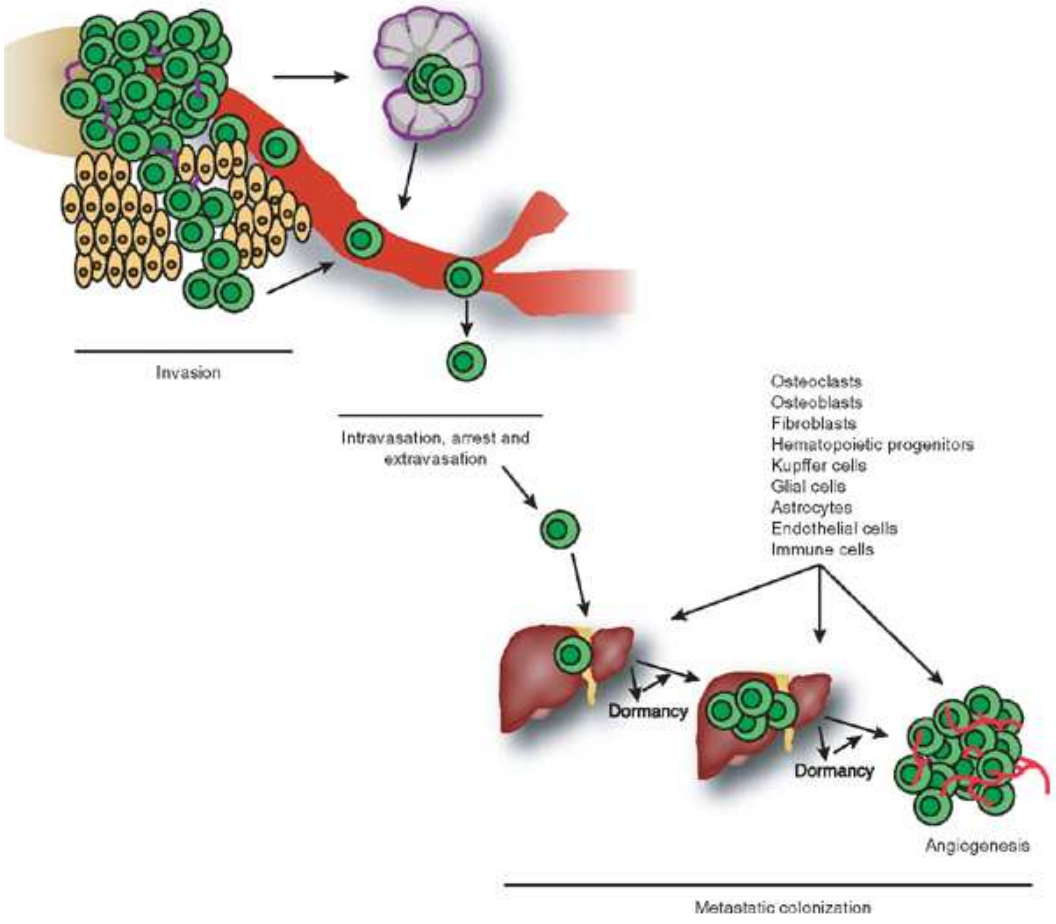


Figure 7. The tumour metastatic process (From Patricia S Steeg, 2006)

At the primary tumour site, tumor cells invade into the lymphatics or directly into the circulation. Once in the bloodstream, tumour cells must survive and avoid immune attack to extravasate. Arrest is most often by size restriction in capillary beds but can involve specific adhesive interactions. The process by which tumour cells form micrometastases and then progressively growing, vascularized macrometastases in a distant organ is termed metastatic colonization. Metastatic colonization involves reciprocal interactions between tumour cells and the microenvironment of the distant organ, and can pause for periods of dormancy. One of the most enduring observations in metastasis research was published in 1889 by Stephen Paget. Describing tumour cells as the “seed” and the host environment as the “soil,” Paget hypothesized that their interaction determines metastatic outcome: “When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil”. This observation predicted that the tissue

environment, composed of a myriad of specialized cell types, extracellular matrices and cells recruited to the site, may facilitate tumour metastasis and contribute to the organ selectivity sometimes seen in metastatic colonization. Complex and redundant pathways involving the tumour cell and the microenvironment mediate tumour invasion at the primary site, survival and arrest in the bloodstream, and progressive outgrowth at a distant site.

Understanding these pathways and their dynamic interactions will help identify promising molecular targets for cancer therapy and key obstacles to their clinical development.

1.8. Soft Tissue Sarcoma (STS)

Sarcomas are a heterogeneous group of rare tumours that arise predominantly from the embryonic mesoderm. The various sarcomas include bone sarcomas (osteosarcomas and chondrosarcomas), Ewing's sarcomas, peripheral primitive neuroectodermal tumours, and soft tissue sarcomas, which are the most frequent. In 2004, approximately 8,680 new cases are expected to be diagnosed in the United States, and 3,660 deaths from soft tissue sarcomas are predicted, accounting for 0.63% of all cases and 1.15% of deaths from cancer (Jemal et al, 2004). Currently, more than 50 histologic types of soft tissue sarcoma have been identified (**Table 3**), but the most common are malignant fibrous histiocytoma (28%), leiomyosarcoma (12%), liposarcoma (15%), synovial sarcoma (10%), and malignant peripheral nerve sheath tumours (6%; Coindre et al., 2001). Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood.

Table 3. Histologic Subtypes of Soft Tissue Sarcoma

Histological Subtypes	n	%
Malignant Fibrous Histiocytoma	349	28
Liposarcoma	188	15
Leiomyosarcoma	148	12
Unclassified sarcoma	140	11
Synovial sarcoma	125	10
Malignant Peripheral Nerve Shealth Tumor	72	6
Rhabdomyosarcoma	60	5
Fibrosarcoma	38	3
Ewing's Sarcoma	25	2
Angiosarcoma	25	2
Osteosarcoma	14	1
Ephiteliod Sarcoma	14	1
Chondrosarcoma	13	1
Clear cell Sarcoma	12	1
Alveolar Soft Part Sarcoma	7	1
Malignant Hemangiopericytoma	5	0,5

Modified from Coindre et al.

As for most types of tumours, the presence of metastasis at diagnosis, or the evolving of such lesions with time, catastrophically reduces the probability of survival. Factors predicting the formation of metastasis in soft-tissue sarcoma patients are not known and similarly obscure remains the modes through which metastases form in these individuals.

1.9. Membrane PGs and tumour

One of the most classical experiments demonstrating the importance of cell surface PGs in tumour progression, and in particular the role of HS-carrying PGs, was originally performed by Jeffrey Esko and collaborators (Esko et al., 1988). This study highlighted that cells genetically engineered to be defective in the HS biosynthesis, and thereby harbouring a compositional deficit of their surface HSPGs, failed to form tumours both in vitro and in vivo. These findings were strongly corroborated by comparative analyses of the GAG structures present on the surface of tumour cells with different metastatic potentials (Cattaruzza et al., 2005). In most solid tumours, especially those of epithelial origin, clinically orientated surveys have shown a certain prognostic correlation between the overall levels of surface PGs expression and disease-free survival (**Table 4**).

Table 4. Potential clinical applications of membrane proteoglycans in the field of oncology (358 papers; 1990-2007)

PG	Diagnostic	Predictive/prognostic
SDC1/CD138	Mesothelioma	Carcinoma: breast, oral squamous cell, pancreatic, cervical, small cell lung, prostate, ovarian Hodgkin's lymphoma Myeloma PEL Endometrial cancer Intrahepatic cholangiocarcinoma Mesothelioma
Syndecan-2	Mesothelioma	?
Syndecan-3	?	?
Syndecan-4	?	?
GPC-1	?	Pancreatic carcinoma
GPC-2	?	
GPC-3	Hepatocarcinoma (Melanoma)	Hepatocarcinoma
GPC-4	?	?
GPC-5	?	?
GPC-6	?	?
NG2	MLL ALL	?

With the possible exception of breast carcinoma, the initial dysplastic phases of epithelial tumorigenic involve a pronounced loss of syndecan surface expression, which occurs at both transcriptional and translational level and is believed to be a prerequisite to attain a migratory phenotype (Matsumoto et al., 1997; Day et al., 1999). Subsequently, an accumulation of syndecans in the stromal compartment accompanies an enhanced invasiveness of the malignant cells and a poor prognosis (Bayer-Garner et al., 2000; Wicksten et al., 2001; Zellweger et al., 2003; Barbareschi et al., 2003; Leivonen et al., 2004). Of the four syndecan family members, SDC1 (**Fig. 8)** is the most extensively studied and was the first HSPG to be identified and cloned. SDC1 is down regulated in a variety of cancer tissues (Wiksten, J. P, 2001, Nakanishi, K, 1999) and the loss of SDC1

expression renders the tumour cells less adhesive, thereby increasing their potential to metastasize. Instead augmented SDC1 expression is a hallmark of malignancy in multiple myeloma, several types of lymphomas and certain leukaemia. In B cell lymphomas, cell surface-bound SDC1 assists the cells in their spreading, cytoskeletal reorganization and signal transduction (Lebakken et al., 2000; McQuade and Rapraeger, 2003). This since, in these pathological entities, SDC1 expression seems to inhibit cell–ECM interactions, haptotactic motility and invasion of three-dimensional matrices/cell monolayers (Liebersbach and Sanderson, 1994; Liu et al., 1998; Langford et al., 2005; Spessotto et al., 2001), while favouring cell–cell adhesions through a re-targeted cell surface redistribution to uropods of polarized cells (Børset et al., 2000).

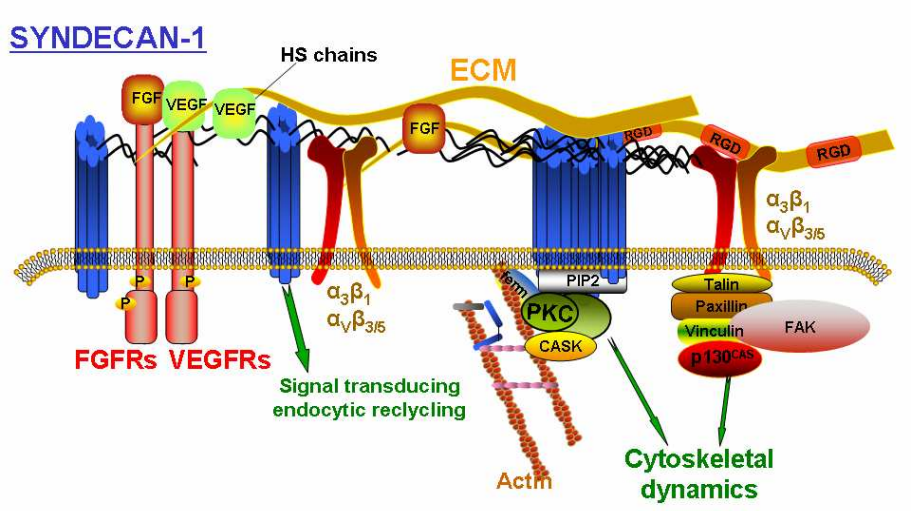


Figure 8. Schematic overview of the putative molecular functions of SDC1. Through its HS chains and core protein the PG may link to ECM components and cooperate with integrins in cell-ECM interactions and/or form oligomeric complexes activating integrin-independent cytoskeletal rearrangements and signal transduction. SDC1 molecules are also endocytosed and recycled through specific pathways that coincidently may activate additional signal transduction events.

Conversely, SDC2 seems to be a characterizing constitutive component of metastatic colon and lung carcinoma cells, where it contributes, alone, or in cooperation with SDC4, to their recognition and stabilization of contacts with ECM molecules serving as potent migratory substrates (Kusano et al., 2000; Contreras et al., 2001; Park et al., 2002; Munesue et al., 2002). The role of SDC2 in cell migration has been less investigated, but several reports indicate that it may positively regulate cell migration, since it is normally highly expressed in cells on migratory conditions (**Fig. 9**). Upregulation of SDC4 has been noted in hepatocellular carcinomas and malignant mesotheliomas and this over expression may correlate with increased tumour cell proliferation (Beauvais et al., 2004).

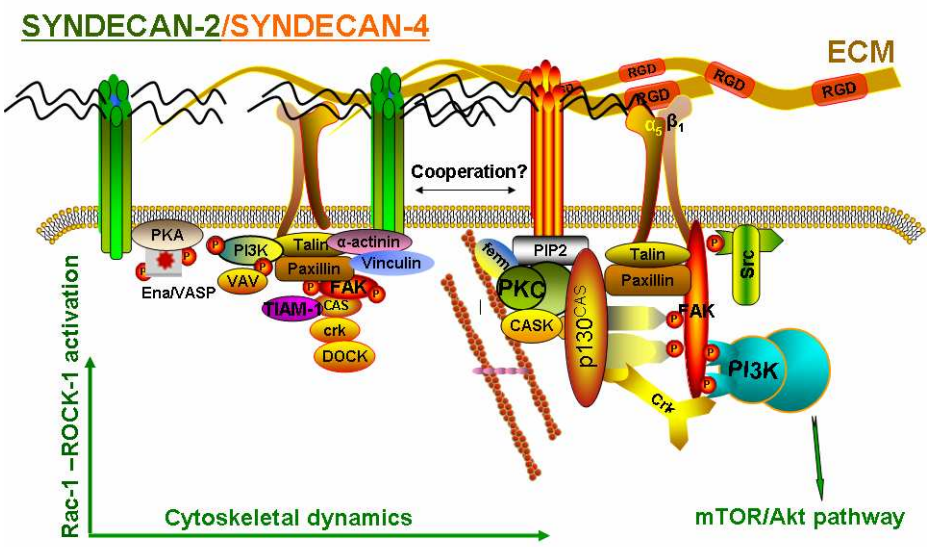


Figure 9. Schematic overview of the putative molecular functions of SDC2 and SDC4. Either individually or through a putative cooperation, SDC-2 and SDC4 strongly impact on cytoskeletal dynamics through phosphorylation events taken place in the cytoplasmic tail. SDC4 cytoplasmic phosphorylation also activates distinct mTOR-Akt survival pathways and endocytic recycling of the two PGs partly contributes to this function, as well as activation of analogous signal transduction pathways.

The role of SDC3 is much less know/studied. SDC3 plays an important role in regulation of skeletal muscle differentiation and development and its multiple functional domains, provide potential sites for mediating the adhesive cell-matrix interactions and cytoskeletal reorganization (Tkachenko E., 2005; **Fig.10**).

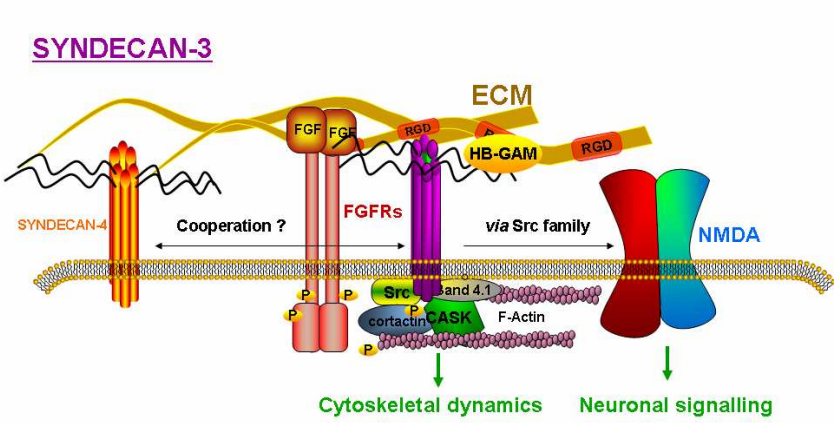


Figure 10. Schematic overview of the putative molecular functions of SDC3. The role of this PG are much less known/studied, but even here a putative cooperation with SDC4 and possibly other syndecans has been proposed. HS chains of SDC3 may exert similar growth factor/cytokine-sequestring and ECM-linking functions as those described for the corresponding HS chains attached to other syndecans. ECM linkage may similarly activate the cytoplasmic tail of the PG and engage the cytoskeleton, as well as other signal transduction events. In the CNS, SDC3 has been proposed to interact with NMDA receptor and participate in the control of neuronal signalling. Nothing is currently known about potential endocytic trafficking of this PG.

In metastatic tumour cells, SDC1 and SDC3 exhibit two cellular routes: an intracellular one involving internalisation of the PG and its occasional translocation into the nucleus (Dobra et al., 2003) and an extracellular one involving surface shedding. The former

translocation routing may reflect a ‘‘functionally unrelated’’ consequence of the loss of cell–cell contacts upon neoplastic transformation, though the precise function of cytosolic and intranuclear syndecans still remains to be defined. It is similarly unknown whether syndecan internalisation is a spontaneous auto-removal process, or whether it is associated with the transformation-induced endocytosis of putative syndecan ligands. On the other hand, release of the extracellular syndecan ectodomain involves a well-known phenomenon of surface shedding driven by MT1-MMP-mediated cleavage of syndecan and having a potentially multivalent role. Through its activation of the Wnt signalling pathway syndecan shedding may contribute to the process of tumorigenic (Alexander et al., 2000) and, via this participation in signal transduction events and the activation of the avβ3 integrin (Beauvais et al., 2004), it may stimulate cell migration (Endo et al., 2003). In addition, highly invasive carcinoma and melanoma cells seem to produce syndecans with a diverse glycanation pattern, which, irrespective of shedding phenomena, confers to these PGs the unique ability to mediate the cells' interaction with laminin(s) (Salmivirta et al., 1994; Engbring et al., 2002). Thus, differential glycosylation of cell surface HSPGs may provide tumour cells with an adjunct to the repertoire of their ECM interactive capabilities needed to accomplish optimal migrations. Glypicans may also exert counteracting roles during neoplastic transformation and tumour progression. The expression of glypicans seems to be involved in cell proliferation and migration and some of these proteins can play a double role acting both stimulating and inhibiting proliferation, in relation to tissue or cellular context (Dietrich et al.,1977). GPC1 expression is up-regulated in pancreatic cancer cells and in breast cancer. Similarly to the situation with SDC1, high GPC3 expression seems to protect transformed cells from converting into a strongly malignant phenotype. This since mammary carcinoma cells engineered to overexpress GPC3 show reduced local invasiveness and develop fewer metastatic lung lesions in murine tumour models (Peters et al., 2003). Accordingly, indirect evidence in the literature suggest that in hepatocellular carcinoma, GPC3 may be released from the developing tumour cells during early phases of their transformation and malignant progression and may be absent from the poorly differentiated, metastatic cells (**Fig. 11)**

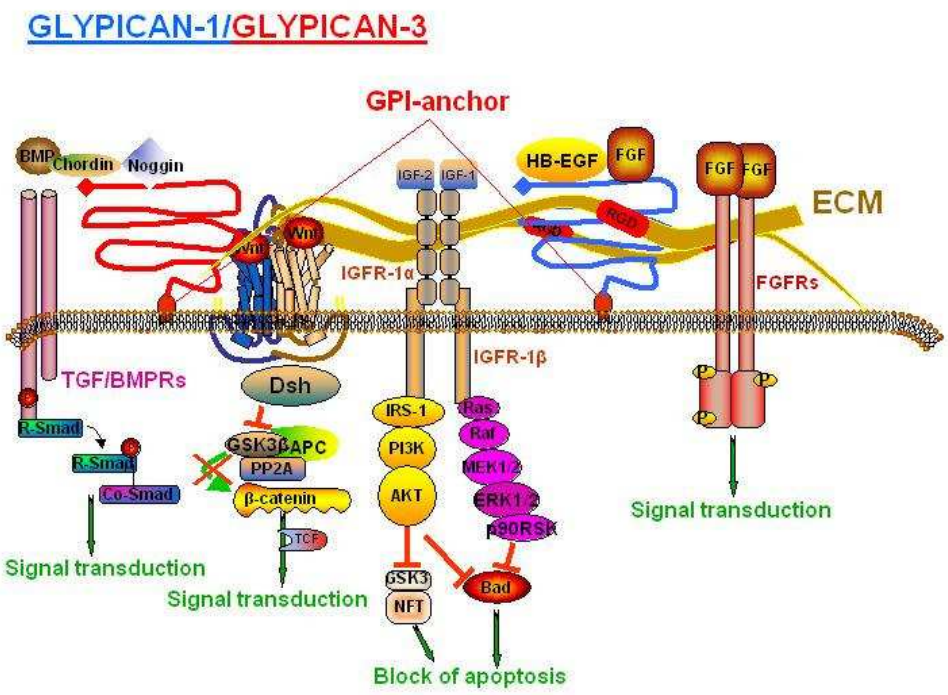


Figure 11. Schematic overview of the putative molecular functions of GPC1 and GPC3. Similarly to syndecans through their HS chains they are believed to sequester a variety of growth factors and additionally have been shown to also immobilize TGFβ and BMP antagonists and Wnt family members, thereby participating indirectly in the control of TGF and Wnt signalling events. There no or very few molecules that are known to specifically associate with the core proteins of these PGs, but their are postulated to be involved in cell-ECM interactions. Endocytosis of GPC1 has been documented and the PG may also act intracellularly. For unknown reasons GPC3 may promote, rather than inhibit apoptosis in certain cell types.

GPC5 has recently been found to be up regulated in rhabdomyosarcomas where it seems to influence malignancy (Williamson et al., 2007; **Fig. 12**).

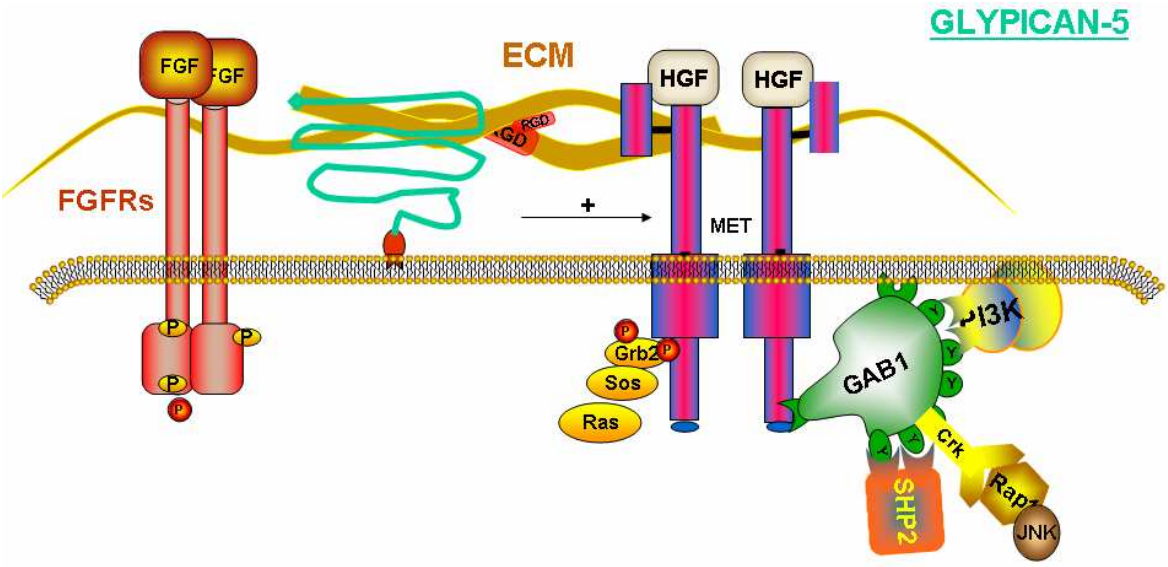


Figure12 . Schematic overview of the putative molecular functions of the GPC5. As for most other glypicans the biological function of the PG is still obscure, but it is the first glypican to be discovered to be upregulated in soft-tissue sarcomas (i.e. rhabdomyosarcomas). Both HGF and FGF family growth factor and ECM linkage are presently the candidate functions of the GPC5.

Conversely, virtually nothing is known about the role of other, i.e. GPC2, 4 and 6 in tumour development. In highly aggressive neoplasia of the nervous system and in melanoma, NG2 seems to play a fundamental role in conferring to the cells the invasive and metastatic potential (Chekenya et al., 2002). In fact, if NG2-negative melanoma cells are transduced with NG2, their ability to give rise to lung metastasis is substantially increased (Burg et al., 1998). It is also expressed by a number of different types of tumours, including melanoma (Real et al., 1985), glioblastoma (Schrappe et al., 1991), chondrosarcoma (Leger et al., 1994), and myeloid leukemia (Smith et al., 1996). NG2 appears to be important for potentiating cell motility (Burg et al., 1997,1998: Fang et al., 1999; Eisenmann et al., 1999) and for modulating cellular responses to growth factors (Grako et al., 1995, 1999; Nishiyama et al., 1996b; Goretzki et al., 1999), properties which are critical for the proliferation and migration of both immature progenitor cells and tumour cells. Instead, NG2 interactions with ECM molecules regulate signalling pathways involved in cell proliferation and motility (Iida et al.,1995 ; Tillet et al., 2002; **Fig. 13**). Linkage of NG2 to ECM components (and its putative interaction with cell surface molecules) triggers cytoskeletal rearrangements (Majumdar et al, 2003) that may, in part, be mediated by the multi-PDZ domain adapter molecule MUPP1. In addition, PKCa-mediated phosphorylation of the threonine residue at position 2256 of the cytoplasmic NG2 tail allows the clustering of the PG at filodopial tips (Lin et al., 1996a,b). Simultaneous molecular interactions involving the cytoplasmic portion of NG2 and the formation of integrin-mediated focal adhesions trigger FAK phosphorylation and down-stream activation of Rac-1 and cdc42 to further control actin microfilament and microtubular dynamics in the filopodial and lamellopodial protrusions of the migrating cell (Majumdar et al, 2003). These signalling cascades also involve activation of the kinase Ack-1 and a FAK-independent activation of ERK.

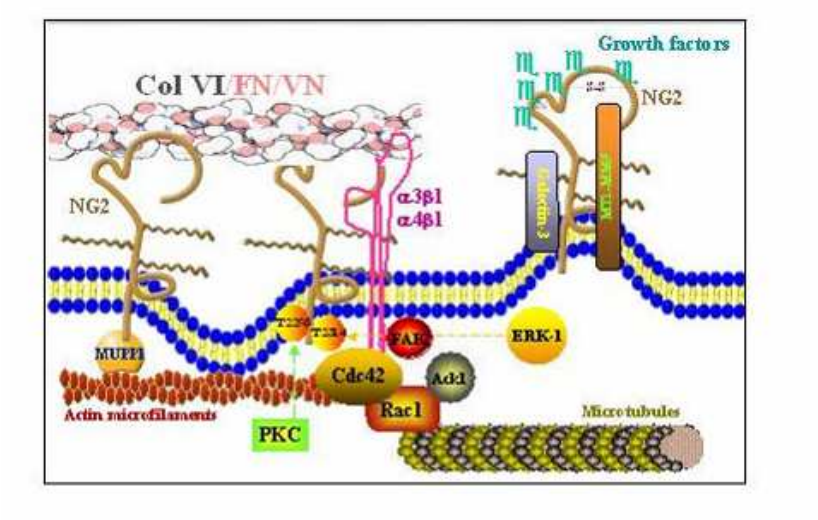


Figure 13. NG2 involvment in regulation of adhesion, migration and invasion of tumour cells. During cancer cell migration, seems to be the underpinning for the FAK/p130cas-mediated activation of cdc42 and Rac1 implicated in the control of

microtubular dynamics. NG2 may also modulate the activity of certain integrins, in particular α3β1 and α4 β1 and electively link to ECM components, where the primary ligand is collagen type VI.

The proposed tumour stimulating action of the NG2 is associated with its ability to function as a docking receptor for PDGF-AA (Stallcup, 2002) and various members of the FGF family (Cattaruzza et al., 2008). It may be potentiate the effect of growth factors by sequestering them at the cell surface and presenting them to their respective signalling receptors. Also matrix metalloproteinases (MMPs) are another ligand for NG2. In particular membrane-type 3 matrix metalloproteinase (MT3-MMP) forms a complex with NG2 that is critical for the ability of melanoma cells to degrade and invade a type I collagen-containing matrix (Iida et al., 2001). However, NG2 is also strongly implicated in the neoangiogenic process by sequestering angiostatin and supporting pericyte sprouting and tubular formation (Goretzki et al., 2000; Chekenya et al., 2002; Ozerdem et al., 2003; 2004; Virgintino et al., 2007a; b) but the precise mode by which NG2 confers to tumour cells invasive and metastatic capabilities and, thereby, influences progression of tumours remains to be fully clarified. By virtue of its direct association with the actin cytoskeleton (Lin et al., 1996a; b; Fang et al., 1999; Barritt et al., 2000), a possible mechanism by which NG2 may affect tumour development and spreading is by promoting the tumour cells interplay with neighbouring host cells and ECM constituents. This function is believed to be exerted through, on one hand, an association of the NG2 with galectin-3 and integrins α3β1 and α4β1 (Iida et al., 1992; 1995; Eisenmann et at., 1999; Fukushi et al., 2004), and on the other by assisting tumour cells in their linking to specific collagens, such as collagen type VI (; Stallcup et al., 1990; Nishiyama and Stallcup, 1993; Burg et al., 1996; 1997; Tillet et al., 1997; 2002), produced endogenously or by the stromal compartment. In mesenchymal tumours collagen type VI transcription seems to be down-regulated as a consequence of neoplastic transformation (Schenker and Trueb, 1998), whereas it is upregulated in the stromal tissue of breast cancer lesions (Iyengar et al., 2005). Remodelling of collagen type VI-containing matrices may contribute to the acquisition of drug resistance in ovarian tumours (Sherman-Baust et al., 2003), probably because it counteracts the drug-activated apoptotic machinery of the cells and triggers intracellular signals responsible for cell survival (Rühl et al., 1999a; b). It remains, however, unknown whether Col VI also promotes tumour spread and metastasis formation and, if so, through which mechanisms.

1.10. Membrane PGs in sarcoma metastasis

In the last years extensive progress has been made in the identifying of molecules implicated either cell-ECM interactions, cell-cell interactions or in adhesion and motility mechanism (Akiyama et al.1993, Yamada et al., 1995). PGs and integrins are molecules

that are fundamentally important for mediating cell adhesion (Akiyama et al.1993, Albeda et al., 1990, Hynes et al., 1992). In fact PG binding sites are often expressed in close proximity to integrin binding domains within ECM molecules or cell surface adhesion molecules suggest that cellular recognition of the ECM might involve the formation of receptor clusters on the plasma membrane that include both cell surface PGs and integrins. It has been seen that NG2 controls tumour progression in melanoma and sarcoma (Benassi et al., 2009) locally and distantly by accentuating growth responses, by mediating the tumour cell-host microenvironment interaction, by promoting neoangiogenesis, and by conferring enhanced drug resistance. In fact NG2 is highly expressed in melanoma lesions and metastases of soft-tissue sarcomas patients show a >5-fold increase in the expression of NG2 when compared to the primary lesion(s) of these individuals (Benassi et al., 2009). Since NG2 proteoglycan was identified as cell surface ligand for collagen type VI (Stallcup et al., 1990; Midwood and Salter, 2001) we went to consider as interaction of NG2-collagen type VI was implicated in tumour progression. To address this possibility we have examined the NG2 and collagen type VI expression patterns in primitive and secondary lesions of sarcoma patients and addressed the cellular and molecular mechanisms through which the interaction of these molecules may propagate tumorigenesis and induce metastasis formation. Instead, the only one evidence about HSPG involvement in sarcoma tumour is given by GPC5 in rhabdomyosarcoma. It has been seen that amplification of GPC5 in rhabdomyosarcoma and its expression plays a role in tumour development (Williamson et al, 2007). GPC5 might be used for therapeutic intervention, as a potential modulator of multiple growth factors, therapies that reduce the function of GPC5 could affect multiple tumorigenic pathways. Instead potential immunotherapeutic strategies have recently been described involving the use of GPC3 (Motomura et al., 2006) and GPC5 might represent a similarly appropriate target for this approach in rhabdomyosarcoma.

2. Material and Methods

2.1. Cell culture

Estabilished sarcoma and melanoma cell lines (Table 4) A375 cell line were obtained from ATCC and whereas a number of sarcoma cell lines were estabilished from surgical specimens obtained from sarcoma patient tretated at CRO Institute (Centre of Oncologic Reference, Aviano (PN), Italy). All cells were cultured in DMEM (Dulbecco's modified Eagle's medium; 1.0 g/L Glucose; BioWitakker) supplemented with Pen/Strep, 2mM L-Glutamine and 10% (v/v) fetal bovine serum (FBS, Gibco).

Table 4. List of sarcoma cell lines used

CELL LINE	HYSTOTYPE
SK-UT-1	LEIOMYOSARCOMA (uterus)
SKL-MS-1	LEIOMYOSARCOMA (vulva)
Saos2	OSTEOSARCOMA (bone)
143B	OSTEOSARCOMA (bone)
MG63	OSTEOSARCOMA (bone)
SW982	SYNOVIAL SARCOMA (synovium)
HT1080	FIBROSARCOMA (connective tissue)
MES-SA	UTERINE SARCOMA (uterus)
Hs913T	FIBROSARCOMA (metast.to lung)
RD	RHABDOMYOSARCOMA (muscle)
SAR 91266	FIBROSARCOMA
A204	RHABDOMYOSARCOMA (muscle)
DMR-SN-8.4.98	LEIOMYOSARCOMA
NTI-LMS-5	LEIOMYOSARCOMA
GCT	PLEOMORPH MALIGNANT HISTIOCYTOMA
SJRH30	RHABDOMYOSARCOMA (muscle)
NTI-LS-1	LIPOSARCOMA
NTI-LMS-3	LEIOMYOSARCOMA
NTI-MFH-3	MALIGNANT FIBROHISTIOCYTOMA
NTI-PNS-1	PNS SARCOMA
NTI-LMS-3	LEIOMYIOSARCOMA
NTI-MFH-1	MALIGNANT FIBROHISTIOCYTOMA
NTI-MS-1	MULLERIAN SARCOMA
SW872	LIPOSARCOMA
NTI-LS-2	LIPOSARCOMA
NTI-LS-3	LIPOSARCOMA
NTI-LS-4	LIPOSARCOMA
NTI-OS-1	OSTEOSARCOMA
NTI-LMS-6	LEIOMYOSARCOMA

The sarcoma cell line GCT was provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy) and maintained in DMEM medium (1.0 g/L Glucose) with 10% FBS. Non-manipulated, mock-transfected and NG2 stably transduced (Burg et al., 1998) murine B16-F10 melanoma cells were grown in DMEM with 10% FBS. Twenty-one-mer siRNA probes against human NG2, scrambled versions of these probes were synthesized with the Dicer siRNA Generation kit (Gene Therapy Systems Inc., San Diego, CA) as previously described (Cattaruzza et al., 2009). In addition siRNA probes against NG2 and integrin β1 were obtained through Ambion (Austin, TX). Human Umbelical Vein endothelial cell (HUVEC) were isolated from umbelical cords (ARCI Ospedale S.Maria) as previously described (Balconi et al., 1986;). HUVEC were manteined till 4 passage in medium M199 (Gibco) supplemented with Pen/Strep, 20% FBS, 50mg/ml heparin (Sigma) and 50ng/ml endothelial cell growth (ECG, Sigma). Human Iliac Venous and Arterial Endothelial Cells (HIAEC), Human Pulmonary Artery Endothelial Cells (HPAEC) and Human Coronary Artery Endothelial Cells (HCAEC) were cultured in Endothelial Cell Growth Medium MV (Cambrex) supplemented with Pen/Strep, 2%FBS, 0.1ng/ml ECG, 1µg/ml Hydrocortison and 1ng/ml basic Fibroblast Factor . Human Lymphatic Endothelial Cells (HLEC; ScienCell) were cultured in Endothelial Cell Medium (ECM, ScienCell) supplemented with Pen/Strep, 5%FBS and ECG. All the cells were incubated at 37℃ in a humidified atmosphere of 5 % CO_2.

2.2. RNA extraction

Total RNA was extracted from sarcoma cells lines and peripheral blood lymphocytes obtained after informed consent using TRIzol (Invitrogen) according to the manufacturer's instructions. To control no DNA contamination was performed a PCR with primers for RLP41 on reverse-transcribed without RNA as substrate.

2.3. Real time quantitative PCR

Quantitative PCR reaction was performed on cDNA by ABI PRISM 7900 Sequence Detector and ABI PRISM 7900 HT Fast Real-Time PCR System (PE Applied Biosystems), with TaqMan technology. Expression of target genes GPC1, GPC2, GPC3, GPC4, GPC5, GPC6, SDC1, SDC2, SDC3, SDC4 and CSPG4 was quantified using TaqMan Low Density Array (TLDA) based on the fluorogenic 5' nuclease assay (**Fig. 14).** The assay were chosen among thr TaqMan Gene Expression Assay library (**Table 5**) and the TLDA was runned on ABI 7900 HT Fast Real-Time PCR System.

Table 5. Gene expression library list

Gene Symbol	Assay ID
GPC1	Hs00157805_m1
GPC2	Hs00415099_m1
GPC3	Hs00170471_m1
GPC4	Hs00155059_m1
GPC5	Hs00270114_m1
GPC6	Hs00170677_m1
SDC1	Hs00896423_m1
SDC2	Hs00299807_m1
SDC3	Hs00206320_m1
SDC4	Hs00161617_m1
CSPG4	Hs00426981_m1

An equal amount of input cDNA (100ng) was used per reaction and loaded in one of the eight sample-loading port of the card. The amount of target gene was normalized to an endogenous housekeeping gene 18S rRNA and relative to cDNA from human mesenchymal stem cells (hMSC), choosen as sample calibrator.

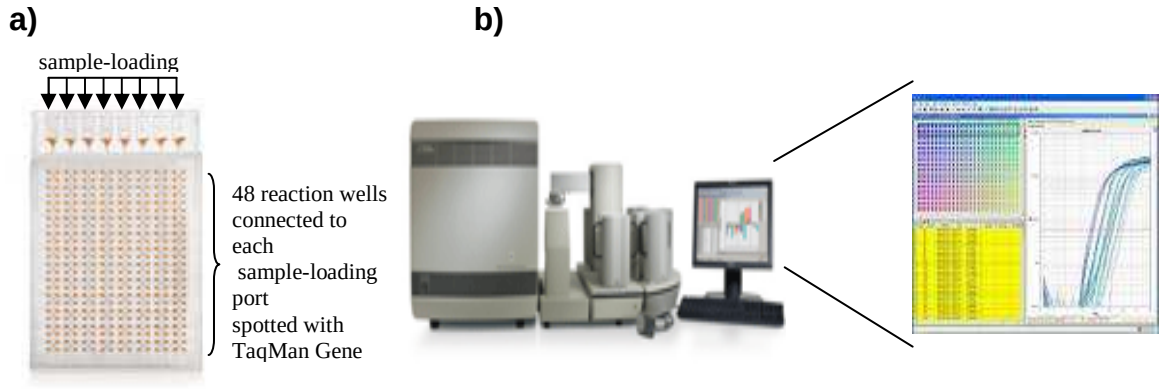


Figure 14. .a)TaqMan Low Density Array; **b)** ABI 7900 HT Fast Real-Time PCR System.

Expression of target genes, NG2 (CSPG4) and α3(VI) chain (COL6A3) was quantified using *TaqMan Gene Expression Assays* (Applied Biosystems) according to manufacturer's protocol and using Assay-on-demand primers with codes Hs99999905_m1 for GAPDH, Hs00426981_m1 for NG2 and Hs00365098_m1 for COL6A3. PCR mixture contained 1.25 µl Target or Endogenous Reference Assay Mix 20X, 22.2ng DNA diluted in 11.25 µl of distillate water, 12.5 µl TaqMan Universal Master Mix 2X (Applied Biosystems) in a 25 µl final reaction volume. Following activation of UNG (Uracil-N-Glycosylase) for 2 min at 50℃ and of AmpliTaq Gold DNA polymerase for 10 min at 95℃ all genes were amplified by 45 cycles for 15 seconds at 95℃ and for 1 min at 60℃. For calcu lation of gene expression we used $2^{-\Delta\Delta CT}$

comparative method. The amount of NG2, COL6A3 was normalized to an endogenous reference (GAPDH) and relative to a calibrator (cDNA from healthy lymphocytes).

2.4. DNA extraction

Clones genomic DNA was prepared using InstaGene^{TM} Matrix 6% (Bio-Rad Laboratories) according to the manufacturer's instructions. DNA quality extraction was done by PCR amplifying the housekeeping gene *RLP41* Forward primer GGAGGCCACAGGAGCAGAAA and Reverse primer TGTCACAGGTCCAGGGCAGA.

2.5. Plasmids and Transfection

143B osteosarcoma cells were stably transfected both with pDisplay GPC-6-HA/pDisplay empy vector and with pEGFP-C1-GPC6/ pEGFP-C1 empty vectors. pDisplay vector contained human cDNA of Glypican-6 Hemagglutinin A (HA)-tagged. The expression vector pDisplay (Invitrogen) containing ORF cDNA of GPC-6 and Hematoglutinin A (HA)-tagged and the box for G418 resistence (Geneticin/Neomycin). It was gently provided by Guido David (University of Leuve-CME). pEGFP-C1-GPC-6 vector was obtained cloning GPC-6 cDNA in pEGFP-C1 by PCR reaction with two specific primer- restriction- adapters 3'-SmaI CAGATCCTCTTCTGAGATGAG and 5' BclI- GAGGGTGATCAGCCCAGCCGGCCAGATCC. The PCR product was cut with BclI (New England Biolabs) and SmaI (New England Biolabs) and purified from the agarose gel using the "QIAquick Gel Extraction Kit" (QIAGEN) in according to manifacture's. pEGFP-C1 vector (BD Biosciences) was linearized with BclI and SmaI for cDNA GPC-6 ligation using T4 DNA Ligase (Biolabs; Pashley C et al., 2003). In order to select stable transfected cells it was necessary to determine the right concentration of G418. For this reason a dose-response curve was performed in 143B cells in response to different concentration of G418, from 100ug/ml up to 1mg/ml. The lower concentration which kill all cells in two weeks was 500 µg/ml. Cells were transfected with pDisplay-GPC6 vector, pEGFP-C1-GPC-6 and their respective empty vectors using metafectene-pro (Biontex, Germany) in according to the manufacturer's instructions. After twenty-four hours from transfection the cells were trypsinized and seeded on 10cm dish at low density (6000cells per dish) and growth in selection condition with G418 (500 µg/ml) for two weeks. For selecting monoclonal cells the colony were picked with p200 Gilson and were expanded; clones were screened by PCR amplification for *Amp* gene Forward primer GTGTCGCCCTTATTCCCTTT and Reverse primer GGCACCTATCTCAGCGATCT. The positive clones were also screened for GPC -6 expression wither by real time PCR and immunonostaining and FACS.

Sarcoma cell lines were also transiently or stably transfected using Lipofectamine Plus as a delivery vehicle with a plasmid containing either the cytoplasmic tail and membrane-spanning

domain of the PG, or the membrane spanning domain plus the entire ectodomain. Human NG2 cDNA clones B, C and D (Plusckhe et al.,1996) were cut with XhoI and SacI, SacI and HindIII, HindIII and BamHI, respectively. These fragments were ligated and inserted into the pEGFP-N1 vector (Clontech Laboratories Inc.), and the sequence of the entire insert comprising bases 4030-7216 of the CSPG4 sequence reported with the NCBI accession number X96753 was verified by automated DNA sequencing. Dominant-negative-like mutant cells named $NG2^{cyto}$ were generated by transient transfection of the cells previously stably transduced to express cells with the 3'-end-directed NG2 siRNA probe 1279 to specifically abrogate the constitutively produced NG2 and spare the transduced deletion construct. Dominant-negative mutants named $NG2^{extra}$ were generated by stable transfection of the same cell lines with a GFP-plasmid containing the entire extracellular portion and the transmembrane domain of human NG2. For this purpose, NG2 cDNA clones H, G and F 29 were cut with XhoI and ApaI, ApaI and BamHI, BamHI and HindIII, respectively, and inserted between the XhoI and HindIII sites in pEGFP-N1 vector (BD Biosciences). Total cDNA from A375 melanoma cells was used as the template to amplify the sequence corresponding to nucleotides 2230-5025, the fragment was inserted into a pGEM-T vector (Promega), and its sequence verified by automated DNA sequencing. The construct was then subcloned into the pEGFP-N1 expression vector containing fragments H, G and F as described above (BD Biosciences). Cells stably transfected with this plasmid were transiently transfected with a 5'-end-directed NG2 siRNA probe (Cattaruzza et., 2008) to differentially eliminate the endogenous NG2 without affecting the transduced deletion construct. In both types of dominant-negative mutants, relative levels of expression of the full-length endogenous NG2, following siRNA knock-down, versus transduced truncated NG2 were determined at the mRNA level by quantitative real-time PCR as described above and by FACS and immunoblotting (Cattaruzza et al., 2009). Deletion constructs lacking different segments of the putative collagen-binding region of the ectodomain were as previously described (Burg et al., 1997; Tillet et al., 1997).

2.6. Western blotting

Cells were solubilized in RIPA lysis buffer (Tris-HCl (pH 7.4) 50mM, NaCl 150mM, P-40 1%, Na-deoxycholate 0.5%, SDS 0.1%, EDTA 2mM, leupeptin 50µM, aprotinin 2µg/ml, soybean trypsin inhibitor 2µg/ml, pepstatinNa_3VO_4 1µg/ml, NaF 1mM, Pefabloc SC 0.8mM) after collection from a 90mm dish and double washing with PBS1X. The protein content in the different samples was determined using Bradford method (Bradford et al., 1976; Zor et al., 1995). Surnatant (400 µl) were incubated overnight in -20℃ with 2.5 volumes of cool 100% methanol. The precipitate was washed in cool 100% acetone and recovered for dissolving in sterile water and 20µl of concentrated media were denatured with 2X sample buffer (final

concentration Tris-HCl (pH 7.4) 0.065M, Glycerol 10.5%, SDS(10%) 21%, Bromophenol Blue(0.05%) 6.5%). Lysates (30 µg) and surnatant concentrated were fractionated by SDS–PAGE using 8% polyacrylamide gels, based upon the expected molecular weight. Supernatant of stable cell lines were collected after 24 hours of culture incubation in DMEM serum free and concentrated utilizing methanol protocol (Burbach et al., 2003). The resolved proteins were blotted to a nitrocellulose membrane (Amersham Pharmacia) by semi-dry electric transfer, and the membranes were blocked for 1 h in TBS buffer (20 mM Tris–HCl pH 7.6, 137 mM NaCl) containing 5% blotting-grade non-fat milk. Membranes were incubated with primary antibodies diluted in milk with 0.1% Tween 20 overnight. The following antibodies were used: anti-HA High Affinity (1:2000; clone 3F10, Roche Diagnostics); anti-GFP (1:5000; Fitzerald); anti-actin (1:3000; Sigma Aldrich). Membranes were washed three times in TBS for 5 min each and then incubated in TBS containing the appropriate horseradish peroxidase conjugated anti-mouse, anti-rat or anti-rabbit secondary antibodies for 1 h. The membranes were washed three times for 5 min each in TBS with 0.1% Tween 20 and then processed for enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham Life Science). Equal loading of samples was confirmed by probing for actin.

2.7. Preparation of ECM substrates

Coverlips and inserts traswell were coating with different ECM molecules (20 µg/ml). Fibronectin, laminin-1 and vitronectin were dissolved in 0.05 M bicarbonate buffer pH 9,6 (Spessotto et al., 2001) for coating the surfaces for about 1 hour a room temperature and 37℃ for vitronectin. Coverslips and the upperside of transwell were washed three time with PBS. Collagen molecules were dissolved in 0.02N of acetic acid for coating coverslips and inserts transwell for about 1 hour at room temperature and left them to dry underflow continuous, in according to the manufacturer's instructions. Whether upper side and under side of transwell were coated with Matrigel (BD Biosciences, San Jose, CA). In the latter case, Matrigel was diluted in cold serum-free DMEM medium at a concentration of 0.5 mg/ml and 50 µl/well dispensed on top of the porous membrane. The Matrigel solution was then incubated for 1 hr at 37℃ to form a semisolid thin gel across the membrane.

2.8. Immunostaining

Sarcoma cells line were seeded in 24 well coated glass coverslips. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and then were washed three times with PBS for 10 minutes. Exogenous GPC6 was detected with anti-HA rat (1:300; Roche) or anti-GFP rabbit (1:500; Sigma), in PBS with 10% NGS and 0.1% Triton-X 100 at 4℃ overnight. Binding of primary antibody was revealed with specific FITC-conjugated secondary antibody, antirat or

antirabbit Alexa 488 (1:100; Invitrogen), in PBS with 10% NGS and 0,1 % Triton-X 100. F-actin was visualized with TRITC conjugated phalloidin (Sigma) for 15 minutes at room temperature. Endosomes was detected using anti-Rab5 (abcam; 1:100), in PBS with 10%NGS and 1%Triton-X 100 overnight at 4℃ and followed by incubation with secondary antibody TRITC conjugated antirabbit (1:100, Sigma) in PBS with 10% NGS and 1%Triton-X 100 for 1 hour at room temperature. NG2 was visualized using anti-NG2 monoclonal antibody, in PBS with 10% NGS, 0.1%Triton-X 100 overnight at 4℃ followed by incubation with TRITC conjugated antimouse (1:100), 10%NGS and Tricton-X 100 for 1 hour at room temperature. Nuclei were counterstained with Hoechst 33258 (Sigma) for 20 minutes a room temperature. Samples were washed three times in PBS and mounted with Mowiol 4-88 (Calbiochem-BD Biosciences) supplemented with 2.5 mg/ml DABCO anti-fading reagent (Sigma-Aldrich).

2.9. FACs analysis

FACS analyses were carried out with the anti-NG2 PE-conjugated antibody 7.1 (Beckman-Coulter) and anti-GPC6 goat antibody (R&D) for 40 minutes at 4℃. The secondary antibody conjugated-PE was left 30 minutes at 4℃. For negative controls, IgG isotypes were used at the same concentration as the primary antibody. Immunosorting of $NG2^+$ and $NG2^-$ sarcoma cells was accomplished MACS-based magnetic bead separation using either the anti-NG2 antibody 9.2.27 (Millipore Corporation) or one of our recently generated anti-NG2 monoclonal antibodies mAb 2161D3 followed by incubation with goat anti-mouse IgG microbeads (Milteny Biotech, Inc). Relative efficiency of the immunosorting procedure and approximate yield of $NG2^+$ cells was on average 23.4% for SW982 cells, 13.6% for HT1080 cells, 28.4% for SK-LMS-1 cells and 18.7% for SKUT-1 cells.

2.10. Cell proliferation assays

For cell growth curves, cells were seeded in triplicate in 6-well culture plates at a density of 1 x 10^5 cells per well. After 24, 48, 72, 96, hours. Cell number and cell viability were determined daily using a Burker chamber (Beckman Coulter-Z2) and the trypan blue dye exclusion test.

2.11. Adhesion and spreading assays

Adhesion of siRNA treated and untreated cells to various purified ECM components was examined using a previously detailed cell adhesion assay denoted CAFCA (*Centrifugal Assay for Fluorescence-based Cell Adhesion*; Spessotto et al., 2001), which allows for both qualitative and quantitative parameters of cell-substratum adhesion to be established. For the assessment of cell attachment to purified ECM molecules, sarcoma cells were seeded in

serum-free medium and allowed to adhere to different substrates for 30 minutes and fixed with 4% of PFA. Subsequently the cells were washed three time with PBS and immunostained. Assessment of cell adhesion to ECM molecules was carried out in multiple independent adopting 15-20 different representative fields of the substrate with discernable matrix deposits. The cells were counted under epifluorescence Nikon eclipse E600 microscope

2.12. Cell Migration assays

FATIMA assay (Fluorescence-Assisted Transmigration, Invasion and Motility Assay) was performed using transwell insert (Falcon HTS FluoroBlock™ Inserts, BD) for evaluating cells migration in response to ECM molecules and a monolayer of endothelial cells. Scratch assay was performed using ibidi Culture-Insert (Ibidi Culture System) for evaluating cells motility. Cell migration, invasion and transendothelial assays were carried out using a modified version of FATIMA (*Fluorescence-Assisted Transmigration, Invasion and Motility Assay*; Spessotto et al., 2001). Transwell inserts (**Fig. 15**) with 8 µm pore size membranes were coated 20 µg/ml using purified ECM molecules as previously described.

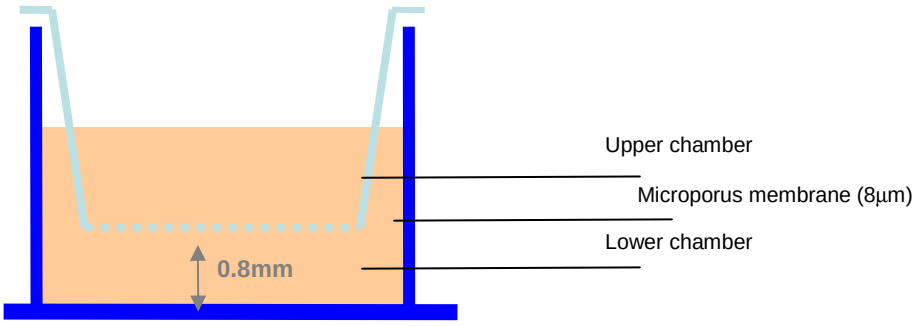


Figure 15. Schematic overview of transwell insert

Cells were previously labeled with Cell Tracker ORANGE and FAST DiTIM (Molecular Probes) in according to the manufacturer's instructions. 100000 cells /ml in 200µl DMEM 1% FBS were seeded on membrane into the upper compartment coated with ECM)molecules. The lower chamber contained DMEM 10%. After 16 hours the cells were fixed in PFA 4% and counted using inverted fluorescent Nikon Eclipse TS 40x. In several experiments invasion assays were performed counting nine fields for each transwell (**Fig. 16**) In others the percentage of transmigrated and "nonmigrated" cells was assessed using the SPECTRAFluor microplate fluorometer (TECAN Group, Maennedorf, Switzerland). Haptotactic migration and invasion assay of siRNA treated and untreated cells in response to purified EMC molecules and Matrigel was examined by real-time videomicroscopy. It was done with an inverted phase contrast microscope (Leica) equipped with an on-stage mini-chamber providing routine incubation conditions (37℃, 5% CO_2). Phase contrast images

were taken in 5-minutes intervals, contrasted digitally and exported into conventional image analyses software for elaboration and presentation.

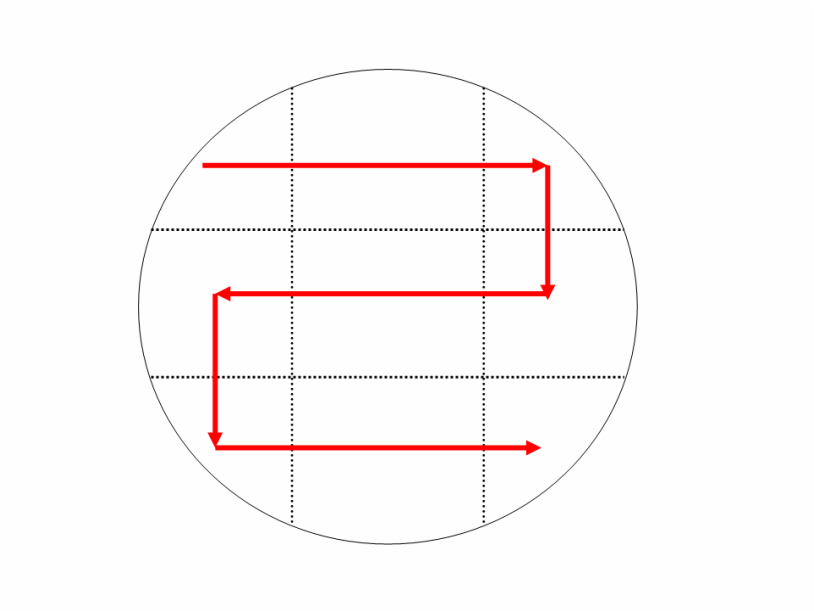


Figure 16. The scheme shows the methodology for counting migrated cells.

Transendothelial assays were performed realizing and endothelial monolayer on the under side of transwell. 300000 endothelial cells for ml previously stained with Orange CellTracker were seeded on the upper side and left in incubator for about 2 hours. Transwells were again reverse in wells of 24 well plate filled with 800µl of endothelial medium. The confluence of the endothelial cell monolayers was reached after 72 hours (**Fig. 17**).

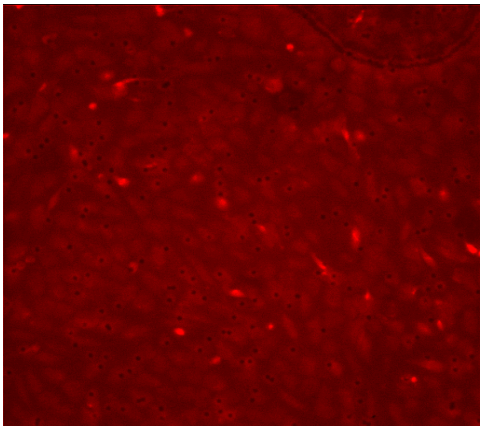


Figure 17. The monolayer of endothelial cells was verified inverted fluorescent Nikon Eclipse TS (40x).

100000 sarcoma cells per ml were previously labeled with Green CellTracker (Molecular Probes) and seeded into the insert. Kinetic curves of migrated cells was performed counting cells in different times using inverted fluorescent microscope.

Scratch assays was performed using Ibidi Culture System for making linear scratches of 200 µm thick. Cells were seeded into each well at concentration of $3x10^5$ cell/ml in 70 µl DMEM 1% FBS and were incubated at 37° and 5% CO_2 C. After about 22 hours the cells reached confluence and the insert was removed by using sterile tweezers with the result of two defined monolayer with a scratch of 200 µm thick (**Fig. 18**).

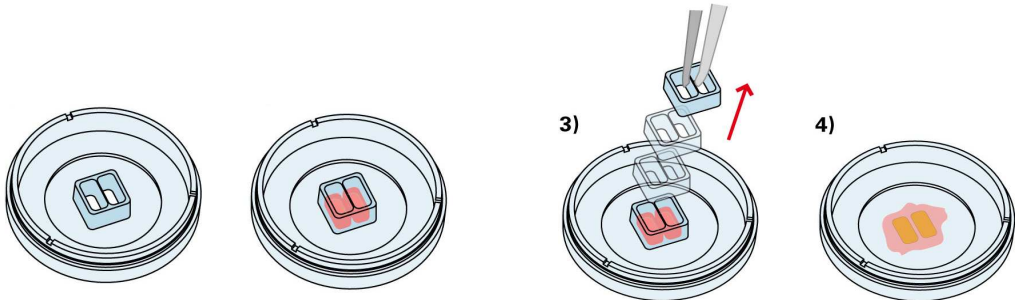


Figure 18. Steps for the Wound healing assays using Ibidi Culture System.

The wounds are observed using inverted phase contrast microscopy. Images were taken using 10x magnification at regular intervals over the course of 12–24 hours. Images were analyzed by digitally drawing lines (using Adobe Photoshop) averaging the position of the migrating cells at the wound edges. The cell migration distance was determined by measuring the width of the wound divided by two and by subtracting this value from the initial half-width of the wound (Valster et al., 2005).

3. Results

3.1. Constitutive PG mRNA pattern

In order to understand the implication of PG surface profiles in the malignancy traits of established cell lines and cells isolated in the our laboratory a screening was performed on 31 sarcoma cells for defining the constitutive pattern of NG2, glypican and syndecan. PG surface were revealed in each sarcoma cell were showed to have a different and particular PG pattern (**Table 6**).

Table.6. PG Expression pattern in sarcoma cells.

Cells	CSPG4	GPC1	GPC2	GPC3	GPC4	GPC5	GPC6	SDC1	SDC2	SDC3	SDC4
GCT	+	+					+	+		+	+
HT1080	+	+			+	+		+	+	+	+
Hs913T	nd[1]	+						+		+	+
NTI-FS-1	+	+				+	+	+	+	+	+
MES-SA		+	+			+		+		+	
SK-LMS-1	+	+					+	+		+	+
SK-UT-1	+	+				+		+		+	+
NTI-LMS-1										+	+
NTI-LMS-3	nd	+				+	+	+		+	+
NTI-LMS-5		+			+		+	+	+	+	+
NTI-LMS-6	+				+	+			+	+	+
NTI-LMS-8	nd	+		nd	+		nd	+	+	+	+
SW872	nd	+					+	+		+	+
NTI-LS-1	+	+						+	+	+	+
NTI-LS-2									+		
NTI-LS-3		+				nd	+	nd		+	+
NTI-LS-4	+	+			+		+	+	+	+	+
NTI-LS-5	+	+						+	+	+	+
NTI-MFH-1	nd	+					+	+		+	
NTI-MFH-3	nd	+					+			+	+
NTI-MFH-4	+							+	+		+
NTI-MS-1				+	+	+	+	+	+	+	+
143B	+	+	+		+	+	+	+	+	+	+
MG63	+							+	+		+
SAOS2	+						+		+	+	+
NTI-OS-1	+		+	+	+	+	+	+	+	+	+
RD	+			+	+			+	+	+	+
A204	nd	+					+	+		+	+
SJRH30	+	+	+	+	+	+	+	+	+	+	+
SW982	(+)[2]	+				+	+	+		+	+
NTI-PNS-1	nd	+				+	+	+		+	+

([1] nd: not determined; [2] (+): to confirm)

This first qualitative analysis were showed an average of 5-8 PGs surface in fibrosarcoma cells. NG2, GPC1, SDC1, SDC3 e SDC4 were always expressed whereas GPC2 and GPC3 were not revealed and GPC4 was expressed only in HT1080 cells. An average of 6-7 PGs surface were identified in leiomysarcoma, SDC3 and SDC4 were always expressed whereas GPC2 and GPC3 were not detected; NTI-LMS-1 cells express only two genes (SDC3 and SDC4). In liposarcoma cells, GPC2, GPC3 and GPC5 were not

expressed and GPC4 and SDC2 were expressed respectively only in NTI-LS-4 cells . An average of 4-5 PGs surface was detected in malignant hystocitoma. GPC2, GPC3, GPC4 and GPC5 were not expressed and NG2 was detected only in NTI-MFH-4 cells. In osteosarcoma hystotype 10 PG on total of 11 was identified in 143B and NTI-OS-1 cells even if have got a different genetic profile; whereas NG2, SDC2 and SDC4 were always expressed. In rhabdomyosarcoma, NG2, SDC2 and SDC4 were always expressed whereas 11 PGs were detected in SJRH30 cells.

Sarcoma cells were tumor of mesenchymal origin and their PG expression were compared to cDNA of hMSC cells (calibrator sample) after normalizing to endogenous housekeeping gene 18S rRNA. The relative expression of surface PGs were showed in **Fig. 19** with the exception of GPC2, GPC3, GPC5 and SDC1 because they were not exhibited in calibrator sample.

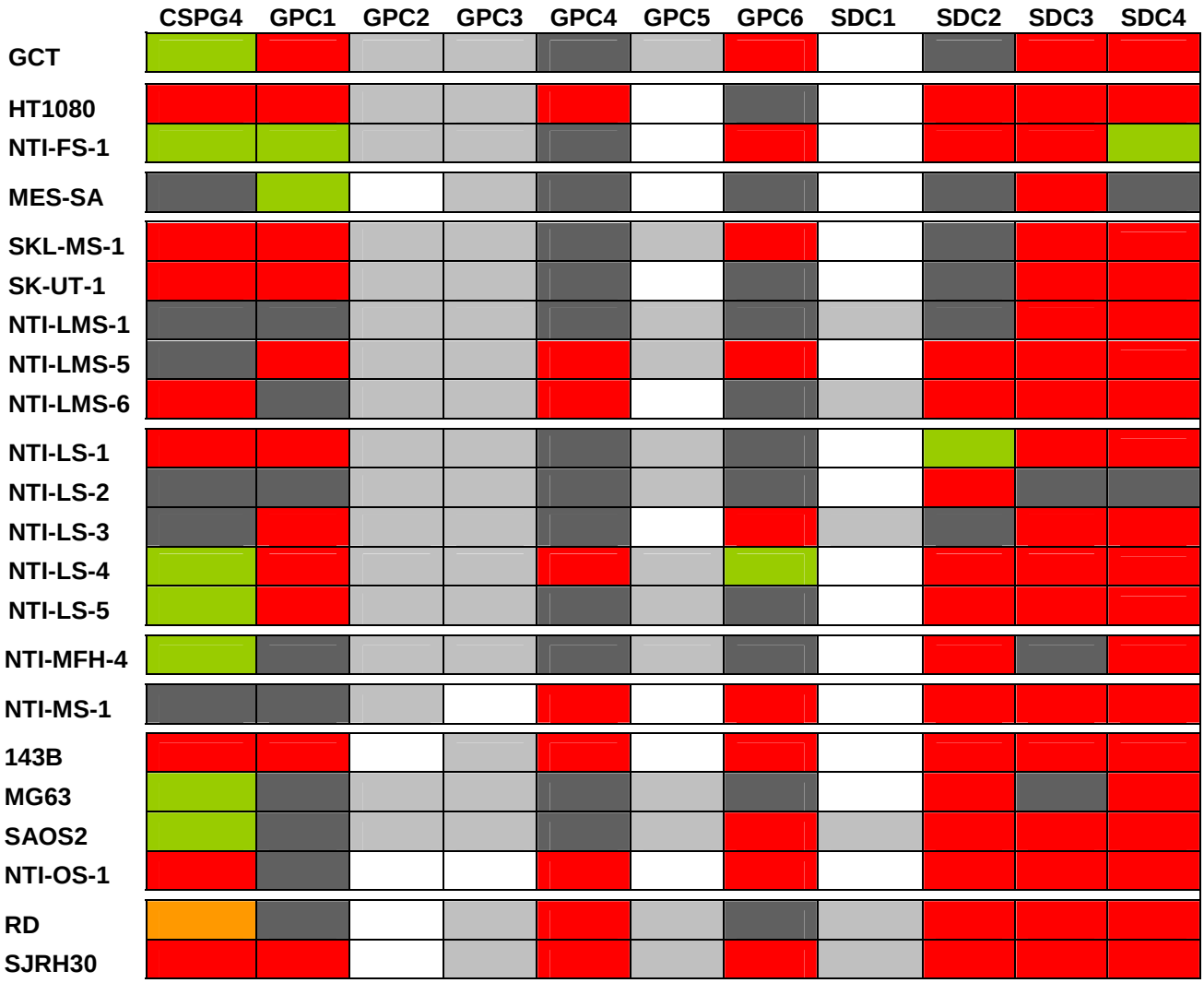


Figure 19. Relative gene expression of sarcoma cells grouped in hystotypes. TaqMan low-density arrays results: gene expression level down-regulate , up-regulate or unchanged in comparison to hMSC as sample calibrator; indicate that PG was not expressed, indicate that PG was not expressed in both cells and sample calibrator, indicate that was not possible to calculate relative expression.

The comparison of the PGs relative expression showed different PGs pattern respect to reference sample in fibrosarcoma cells, in fact all PGs were down regulated in HT1080 cells whereas NG2, GPC1 and SDC4 were up regulated in NTI-FS-1 cells. In leiomyosarcoma cells all PGs expressed are down regulate and NG2 expression was 50% less than in the sample calibrator (data no show). In liposarcoma cells, NG2 expression was up-regulated in NTI-LS-4 and NTI-LS-5 cells, GPC6 and SDC2 were up regulated respectively in NTI-LS-4 and NTI-LS-1 cells, instead the other PGs were down regulated in comparison to calibrator sample. In osteosarcoma cells all PGs were down regulated with the exception of NG2 that was up regulated in MG63 and Sa-os-2 cells respect to sample calibrator.

3.2. Model cells with diverse pattern of surface PGs

This preliminary screening has permitted us to identify 24 different model cells (**Fig. 20**) with diverse combinations of surface PGs. It was been found that sarcoma cells may constitutively and coincidently expressed from 4 to 8 of the 11 surface PGs with the exception of NTI-LS-2, 143B and SRJH30 cells.

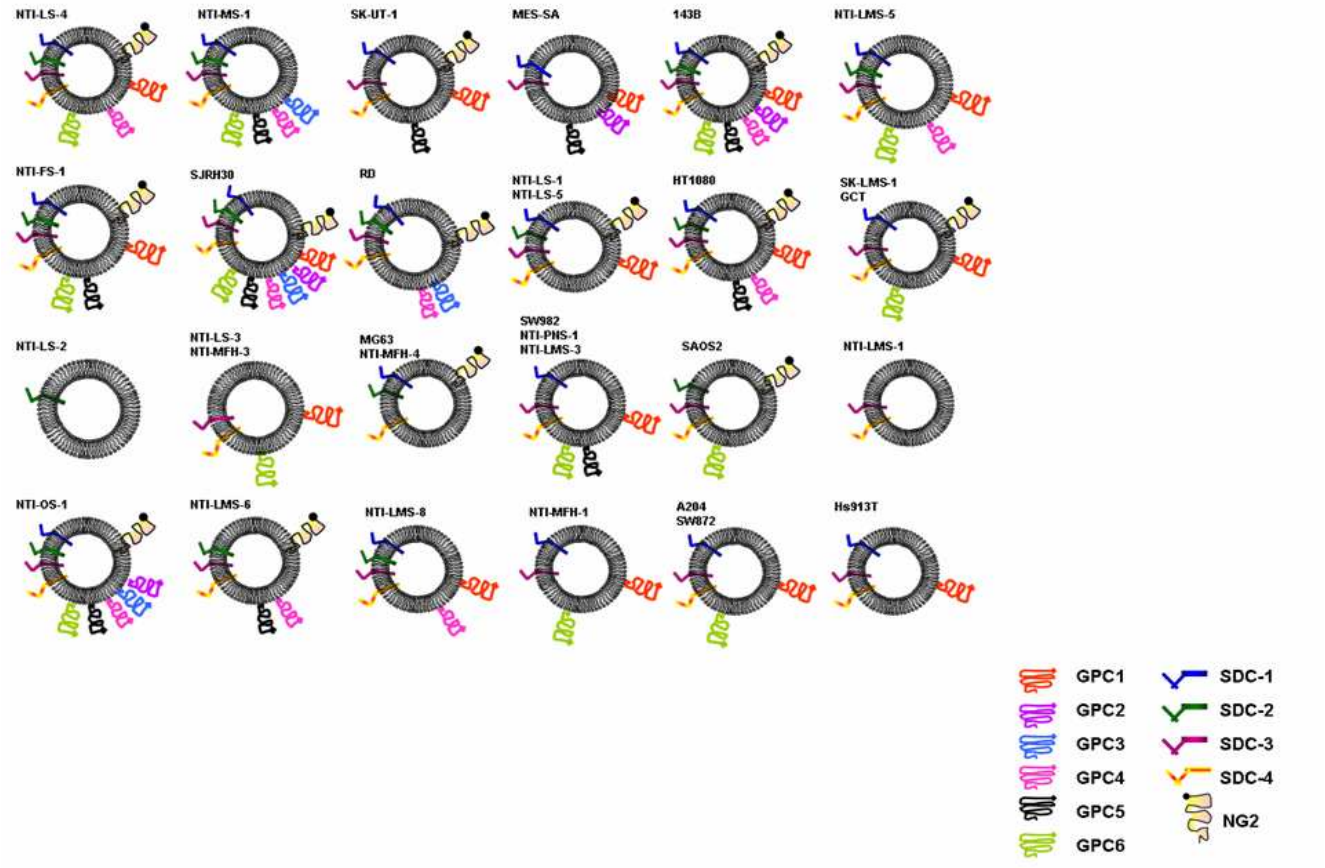


Figure. 20. Overview of 24 model cells characterized by their PG profile

To start to investigate the PG surface profiles as pro- and anti-tumorigenic we delineated strategies to modify the PG expression of 143B osteosarcoma cells by stable gene

transduction and by examining how these modifications affected the cells adhesive and migratory capabilities in response to selected ECM substrates, and endothelial monolayers. For this aim, 143B were stable transfected for overexpressing GPC6 that was chosen as a first PG to modulate since there is currently no knowledge about its involvement in tumor behaviour.

3.3. GPC6 subcellular localization

143B cells were stable transfected with GPC6 using pDisplayGPC6 vector or pEGFP C1-GPC6 vector to produce overepressing cells. GPC-6 N-terminal was fused respectively with HA or eGFP as reporter genes. GPC6 overexpression (143BGPC6+) was detected by qPCR (data not shown), immunostaining and Western blotting (**Fig. 21**).

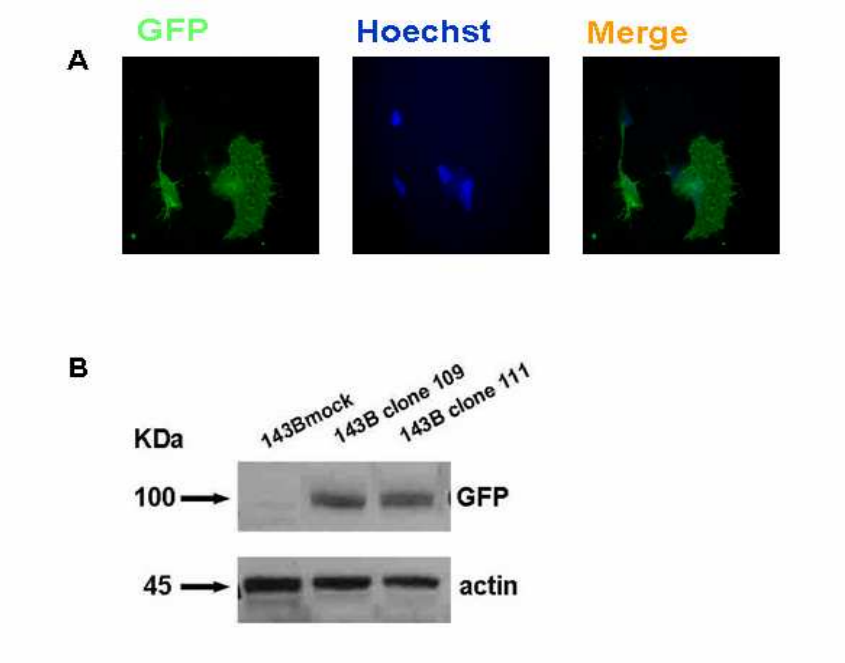


Figure 21 . 143B GPC6 transfected with pEGFPC1GPC6 vector . **A).** GPC6 detection by immunostaining using anti-GFP and the nuclei were counterstained with Hoechst 33258 and examinated by fluorescent microscope; **B).** GPC6 expression together with GFP protein, the fusion product was about 100KDa; 143B mock (control) and two different selected clones 143B clone 109, 143B clone 111; actin was shown as control loading.

A detail analysis of 143B GPC6 overexpressing cells by confocal laser microscopy and flow cytometry confirmed the localization of GPC6 on surface (**Fig.22**).

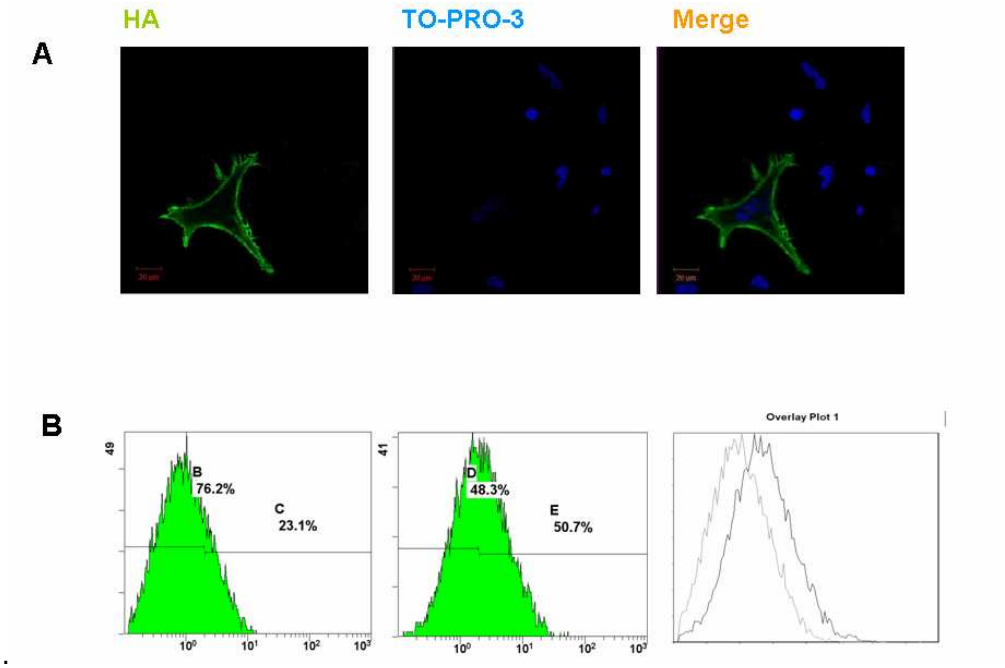


Figure 22. GPC-6 localization on membrane surface**. A).** GPC6-HA detection using immunostaining with anti-HA and nuclei was counterstained with TO-PRO-3. **B).** GPC6 overexpression was detected using anti-GPC6 in comparison with control.

3.4. Effect of GPC6 overexpression on cell morphology

In order to investigate the relationship between GPC6 overexpression and cell shape, the 143B GPC6 overexpressing cells were stained with phalloidin-TRITC to reveal an actin filaments organization (F-actin) (**Fig. 26**).

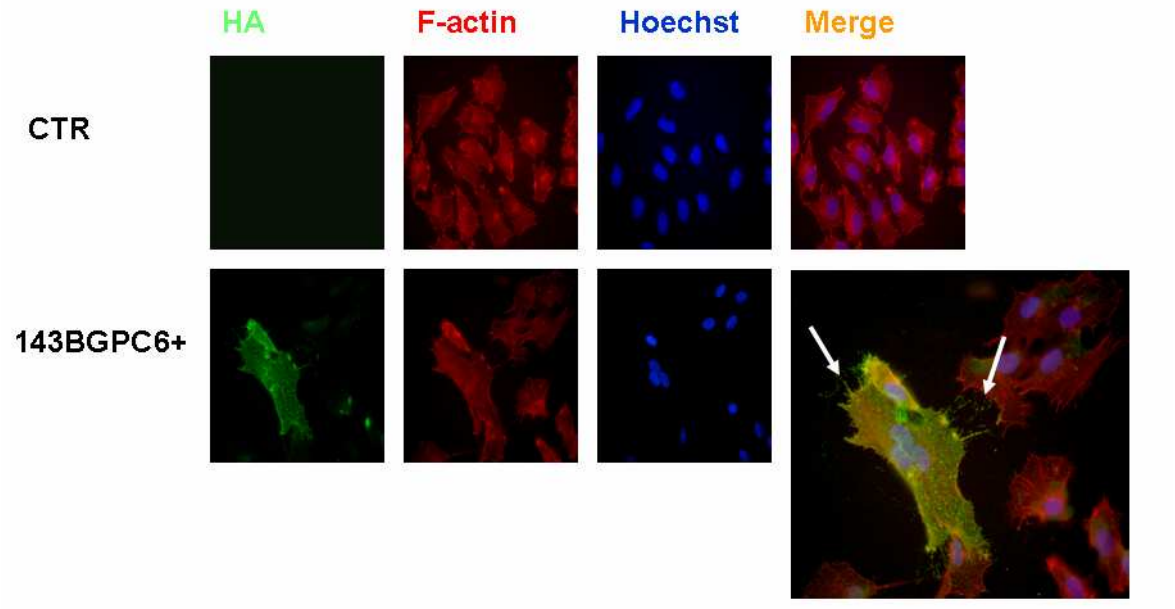


Figure26. Cells morphology was observed with phallodin staining that highlighted actin filament protrusion. GPC6 was detected with anti-HA and nuclei were counterstained with Hoechst 33258 (60x).

143B GPC6 overexpressing cells have showed a markedly different cellular morphology than the vector control cell line. Morphological changes induced by GPC6 overexpression could be associated with changes in the organization of their actin filaments. In particular, phalloidin staining revealed that 143BGPC6 overexpressing cells showed protrusions similar to fillopodia/lamellipodia. Moreover it also possible to observe a more concentrations of F-actin in the attachment sites of the cells to the substrate, suggesting a more pronounced focal contact formation.

3.5. Changes of PG profiles upon modulation of GPC6 overexpression

Modulation of surface PG profile was demonstrated in tumour cells (Timar et al., 1990). To that end, for analyzing forced expression of GPC6 in 143B cells we also investigated the relative expression of PGs surface compared to 143B vector control cells.(**Fig. 27**)

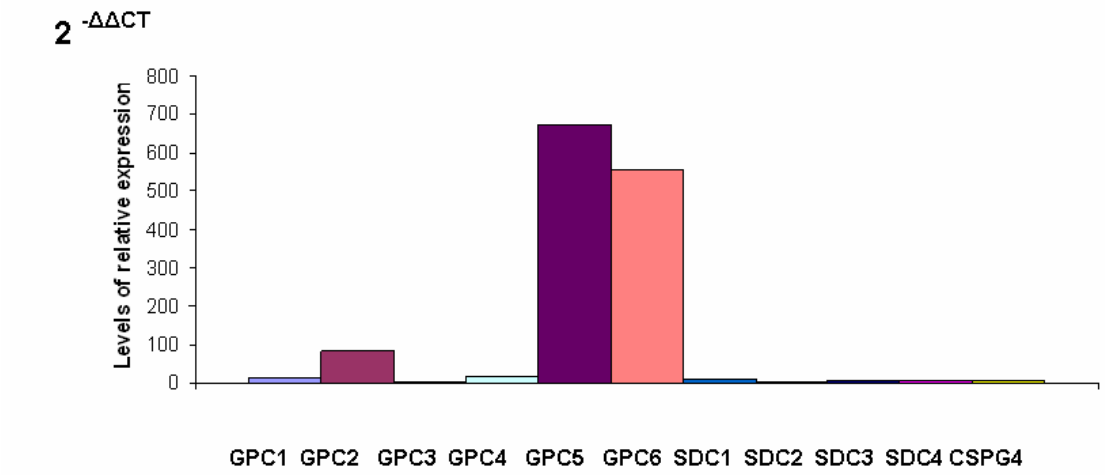


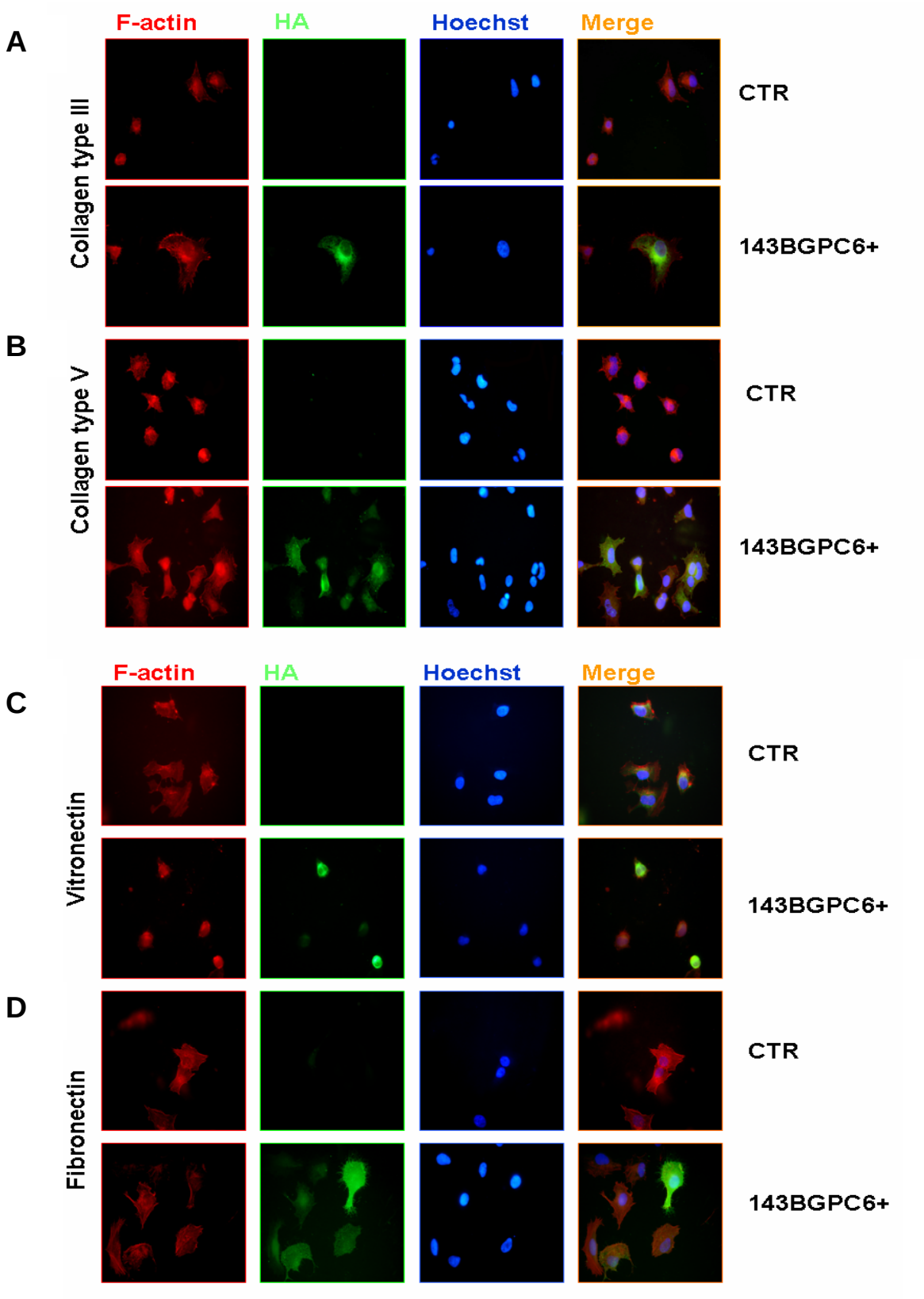
Figure 27. PGs surface modulation by qPCR. The level of relative expression was calculated using 143b vector control cells as calibrator sample after normalizing to endogenous housekeeping gene 18S rRNA

It was observed that the PG surface profile in 143B GPC6 overexpressing cells was modified when compared to control vector cells. First of all 143BGPC6+ showed an evident over expression of GPC6 (about 500 fold) respect to 143B vector control. GPC1, GPC2, GPC4 and SDC1 had a little increased of expression respect to control. Whereas it was interesting to observe an evident over expression of GPC5, about 570 folder than the control. The misexpression GPC6 on a given background of surface PGs brought an evident modulation of the other PGs.

3.6. Role of GPC6 in cell-ECM interactions

Modulation of surface PGs profile and changes in morphology of 143B overexpressing cells had led us to investigate how those alterations could affect the adhesive capabilities of this cells. A preliminary screening was made analyzing the spreading of 143BGPC6

overexpressing cells on different ECM molecules (fibronectin, vitronectin, laminin-1, collagen type III and V). Cell spreading was evaluated observing the distribution of actin filaments staining (F-actin) of 143BGPC6+ cells in response to ECM substrates and uncoated surface (as a control; **Fig. 28**).



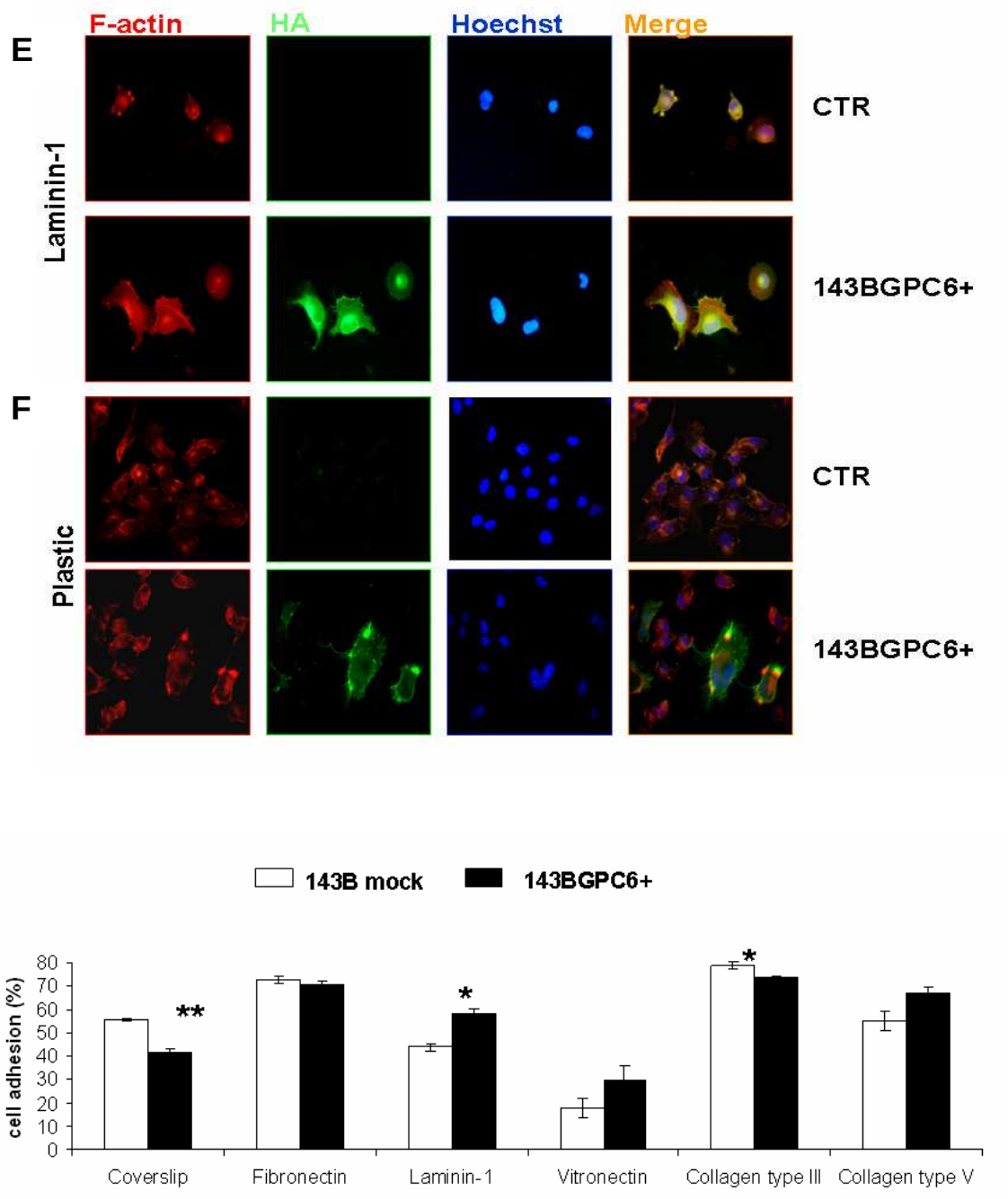


Figure 28. Adhesion and spreading ability of 143BGPC6+ cells on different molecules substrates: collagene type III (**A**), collagene type V (**B**), vitronectin (**C**), fibronectin (**D**), laminin-1 (**E**), uncoated surface (**F**). The percentage of adhesion 143BGPC6+ was compared to 143B vector control. Valuation of cell adhesion was performed counting about 1000 cells in several fields (average of percentage of cells adhesion to the substrate). The assay was made in triplicate (* $p<0,05$; ** $p<0,01$)

The behaviour of 143B overexpressing cells could be concatenated to different surface PGs profile. 143B GPC6 overexpressing cells showed different ability to adhere on each ECM molecules in particular on laminin-1 and collagene III substrates. Moreover the spreading of 143BGPC6+ cells was lowest than the vector control cells on uncoated surface. It was interesting to look the lacking of stress fibres in both cells on vitronectin and uncoated substrates.

3.7. Haptotactic migration of 143BGPC6 overexpressing cells

Migration ability of 143BGPC6 overexpressing cells was evaluated in response to different ECM molecules (fibronectin, laminin-1, vitronectin and collagen type III and V). The upper side of transwell was coated with ECM molecules and the migration of cells was estimated after 16 hours to ensure that migration could not be affected from cell proliferation. The migration of 143BGPC6 overexpressing cells was compared with 143B vector control cells (**Fig. 29**)

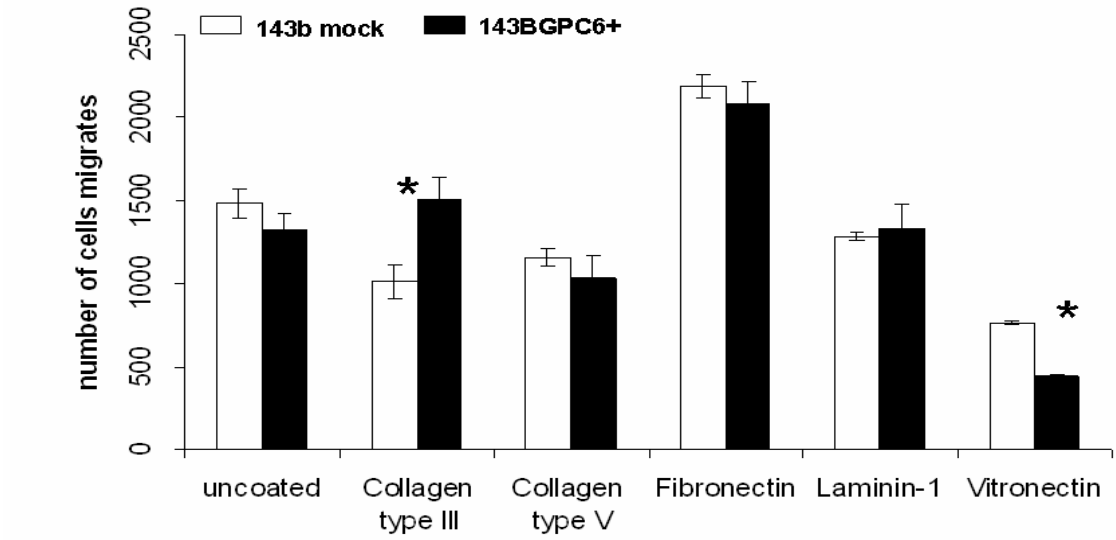


Figure 29. Number of cells migrates in response to ECM molecules. The assay was performed using HTS fluoroblock transwells. Cells were previously labelled with OrangeCell Tracker and the number of cells migrates were counted in nine different fields, using fluorescent inverted microscope (40x). The experiments were made in duplicate (histograms shows an average of experiments in duplicate, * p<0,05).

There was not visualized difference in cells migration but the motility of 143BGPC6 overexpressing cells in response to ECM molecules was resulted to be significantly different on collagene III and on vitronectin substrates.

3.8. Kinetics of cell motility displayed by 143BGPC6 overexpressing cells

To study the motility of 143B GPC6 overexpressing cells was performed a wound healing assay (or scratch assay) using Ibidi Culture System as previously described in material and methods. The wounds were observed using inverted phase contrast microscopy. Images were taken at regular intervals 0, 3, 5, 7, and 24 hours (**Fig.30**) and analyzed for realizing kinetics curve. 143BGPC6+ cells motility was compared to 143B vector control cells

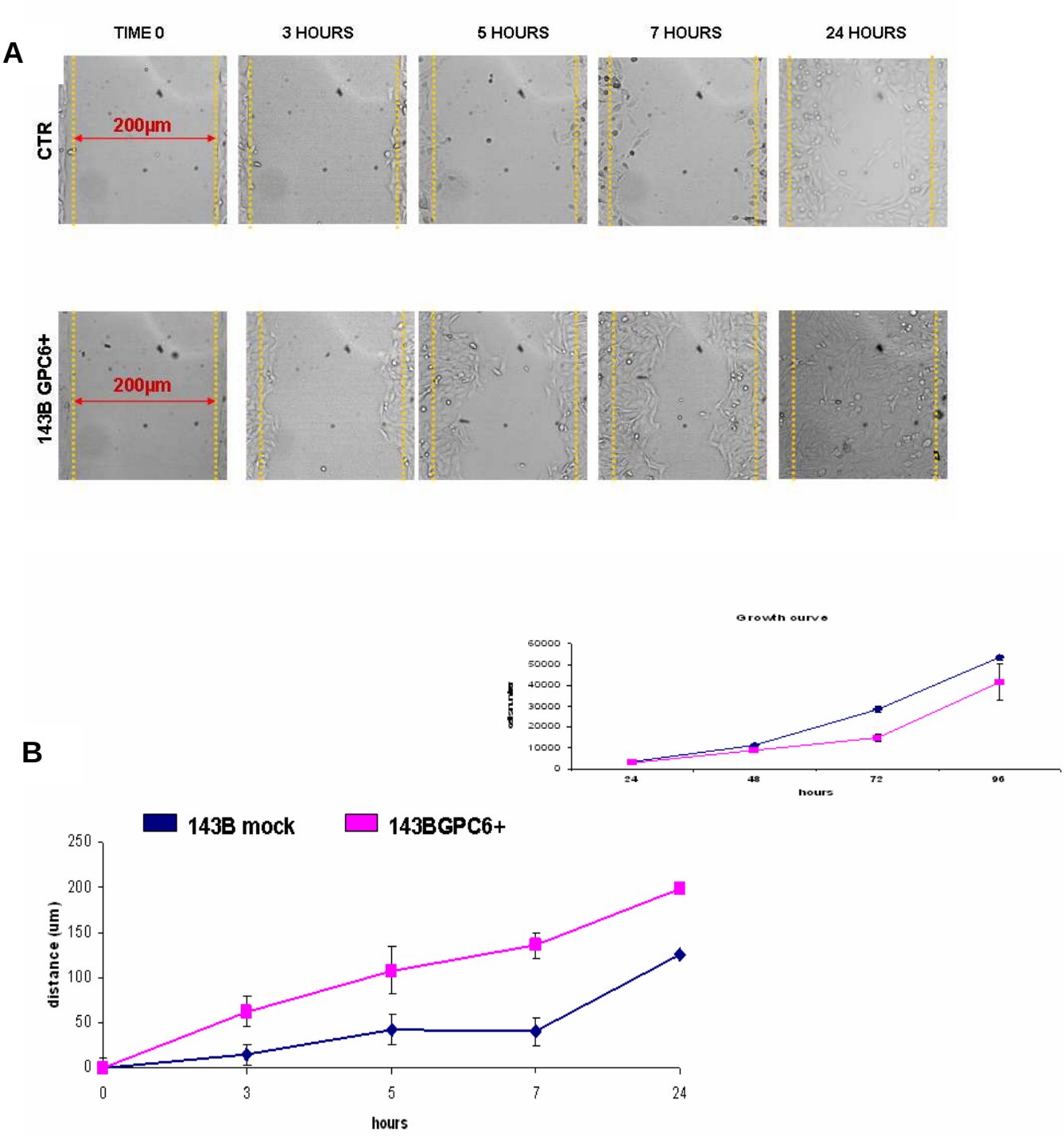


Figure 30. Kinetics migration of 143BGPC6+ cells and control cells. (**A**). The trend of migration was detected using inverted phase contrast microscopy (10x). Scratch assay was made in duplicate and the kinetic curves were showed the same trend; (**B**). Kinetic curves of 143BGPC6+ and control cells (averages was made by measuring the width of the wound in 5 different points of captured images using draw line of adobe Photoshop).

GPC6 overexpression in 143B cells has permitted the closure of the wound more quickly than the control cells. Moreover it was possible to observe how the cell motility was independent from cell proliferation.

3.9. Intracellular fate of transduced GPC6

Modified expression of GPC6 has been observed in 143BGPC6+ cells which could be attributable to shedding or to enhanced endocytocis of the PG. For detecting an eventual proteolysis of GPC6 a preliminary analysis was made going to look the GPC6 expression level in culture medium. The culture medium of 143BGPC6 overexpressing cells was concentrate (clone 109 and 111) and the protein lysate was used as control (**Fig. 31**).

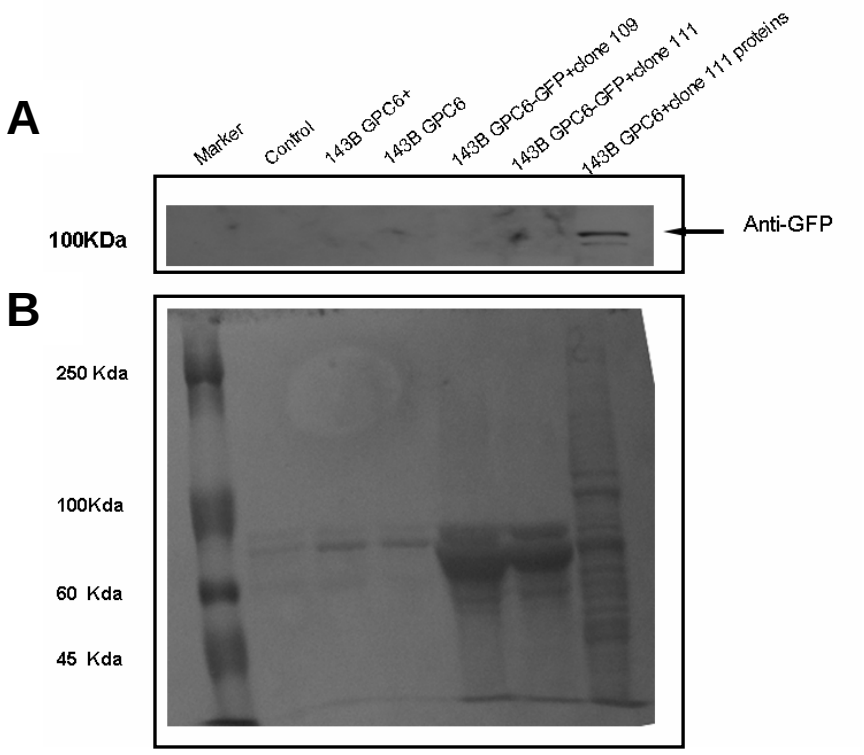


Figure 31. Western blotting of GPC6-GFP protein levels in surnatant cultured cells. **A).** In order to loading surnatant: concentrated of 143B, 143BGPC6+ clone1, 143BGPC6+ clone2, 143BGPC6GFP+ clone 109, 143BGPC6GFP+ clone 111 and protein lysate of 143B GPC6GFP+ clone 111 **B).** Ponceau S staining shows protein loading

From this first approach it was possible to observe the lacking of GPC6 protein in culture medium showing that GPC6 recycling could have another way. Since GPI-anchored protein were internalized and transported in recycling endosomes the fate of GPC6 was studied analysing the endosome vesicles with Rab proteins (Rab-5; **Fig.32**).

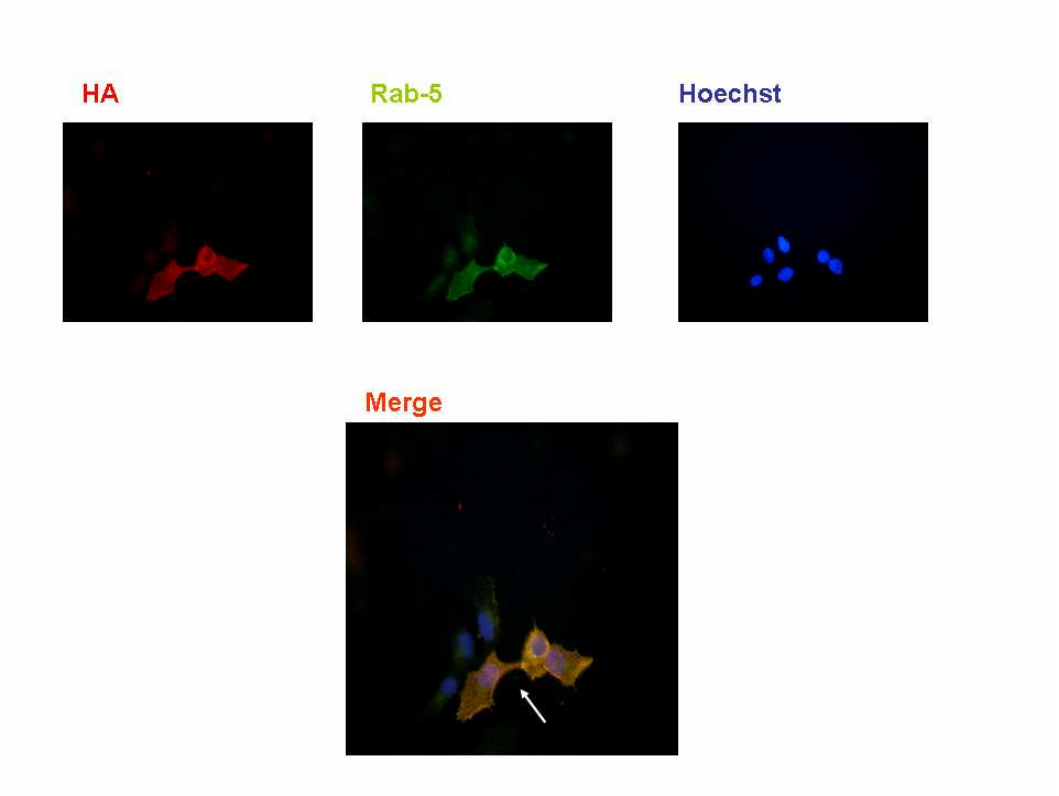


Figure 32. Colocalization of GPC6 and Rab-5 (arrow). There was not Rab-5 expression in cells where GPC6 has ceased to be delivered. Images were taken by fluorescent microscope (60x).

Preliminary analyses showed a correlation between expression of GPC6 and Rab-5 suggesting a high endocytotic activity of GPC6. Rab-5 was not revealed in 143B that not expresses GPC6. This hypothesis will be validated through confocal laser microscope.

3.10. NG2 and collagen type VI expression in sarcoma cells

Since NG2 and collagen type VI cooperate in tumour progression (Stallcup et al., 1990) we have studied how NG2-collagen type VI interactions may influence the behaviour of sarcoma cells. We have screened the expression of α3(VI) (collagen type VI) and NG2 in sarcoma cells by qPCR and FACs for looking if there was a relationship between NG2 and collagne VI expression in sarcoma cells. A375 melanoma cells were used as reference (**Table 8**)

Table 8. Expression of NG2 and collagene VI in sarcoma cells

Cell line	Type of sarcoma	NG2 expression levels (%)[a]	Col VI expression[b]
SK-LMS-1	Leiomyosarcoma	88.5	+
SK-UT-1	Leiomyosarcoma	63.6	+
MG63	Osteosarcoma	83.5	--
SW982	Axillary synovial sarcoma	26.3	--
GCT	Fibrous histiocytoma	47.8	+
HT1080	Fibrosarcoma	37.5	+
143B	Osteosarcoma	61.8	--
SW872	Liposarcoma	99.4	+
RD/KD	Rhabdomyosarcoma	n.d.	--
A375	Melanoma	91.2	n.d.
NT-LS-1°	Liposarcoma	34.1	--
NT-LS-2	Liposarcoma	0.9	--
NT-LS-3	Liposarcoma	1.7	--
NTI-MFH-2	Fibrous histiocytoma	1.0	--
NTI-OS-2	Osteosarcoma	81.2	--
NTI-LMS-1	Leiomyosarcoma	76.8	--
NTI-LMS-4	Leiomyosarcoma	2.3	+
NTI-FS-1	Fibrosarcoma	32.4	+++

Different expression of NG2 was detected in cells, SW872 cells were showed a high expression of NG2 (99,4%) than the other cells. High expression levels were detected also in SK-LM-S-1 (88,5%), MG63 (83,6%), NTI-OS-2 (81,2%) cells. Low levels of NG2 were observed in NT-LS-2 (0,9%), NT-LS-3 (1,7%), NTI-MFH-2 (1,0%) and NTI-LMS-4 (2,3%) cells. Collagen type VI was expressed in SK-LM-S-1, SK-UT-1, GCT, HT1080, SW872 and NTi-LMS-1 cells. High expression levels of collagene VI were detected in NTI-FS-1 cells.

3.11. NG2 involvement in the adhesion and spreading of sarcoma cells on collagen type VI substrates

In order to investigate NG2 involvement in sarcoma cells adhesion on collagen type VI we performed adhesion assays using CAFCA methodology which allows a quantitative analysis of cell-substrate interactions. We have chosen sarcoma cells which exhibited

diverse constitutive levels of NG2 as NTH-LS-2, NTI-FS-1, 143B, NTI-OS-2, SK-LMS-1 and SW872 **(Fig. 33).**

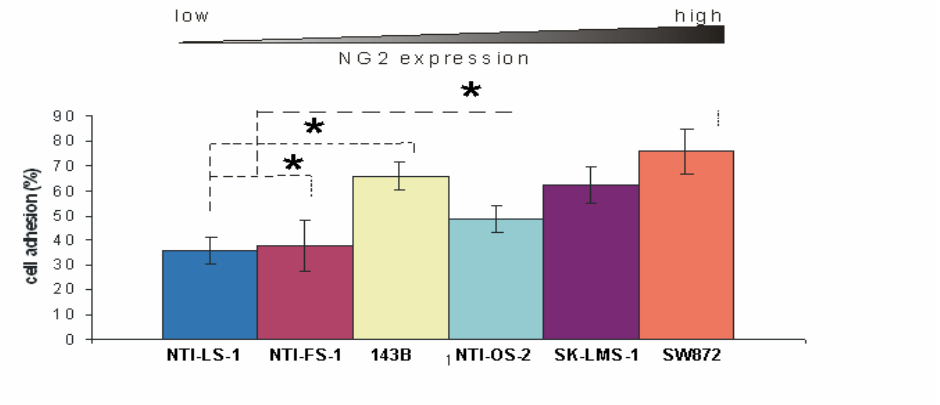
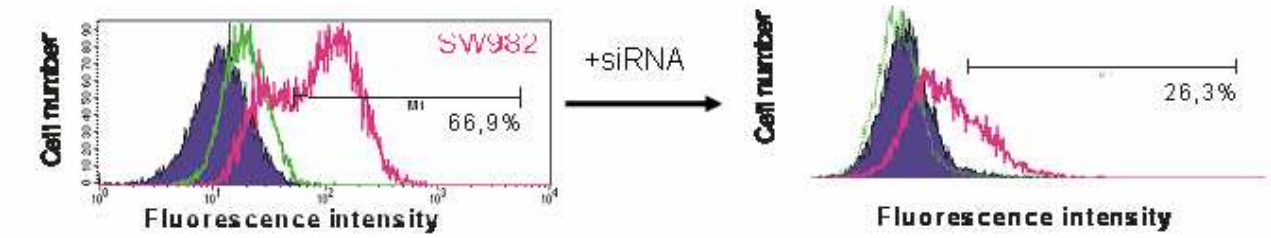


Figure 33. Relative levels of cell adhesion of different sarcoma cells . The comparisons the levels of adhesion with high NG2 expressing cells were found to be significantly higher than those of the low expressing ones ($p<0,001$)

The assay showed a clear relationship between NG2 expression levels and the ability of the cells to bind to collagen type VI substrate. Adhesion of sarcoma cells to collagen type VI was looked to be largely commensurate to their constitutive surface levels of NG2.

For further understanding if NG2 was really involved the binding to collagne type VI, sarcoma cells were NG2 silenced using RNAi; representative levels of NG2 knockdown by RNAi as determined by flow cytometry using the anti-NG2 antibody 7.1 (Cattaruzza et al., 2009; **Fig.34**). CAFCA assay was performed using either NG2 deprived cells or untreated cells on collagen type VI and collagen type I substrates (as a control).

A



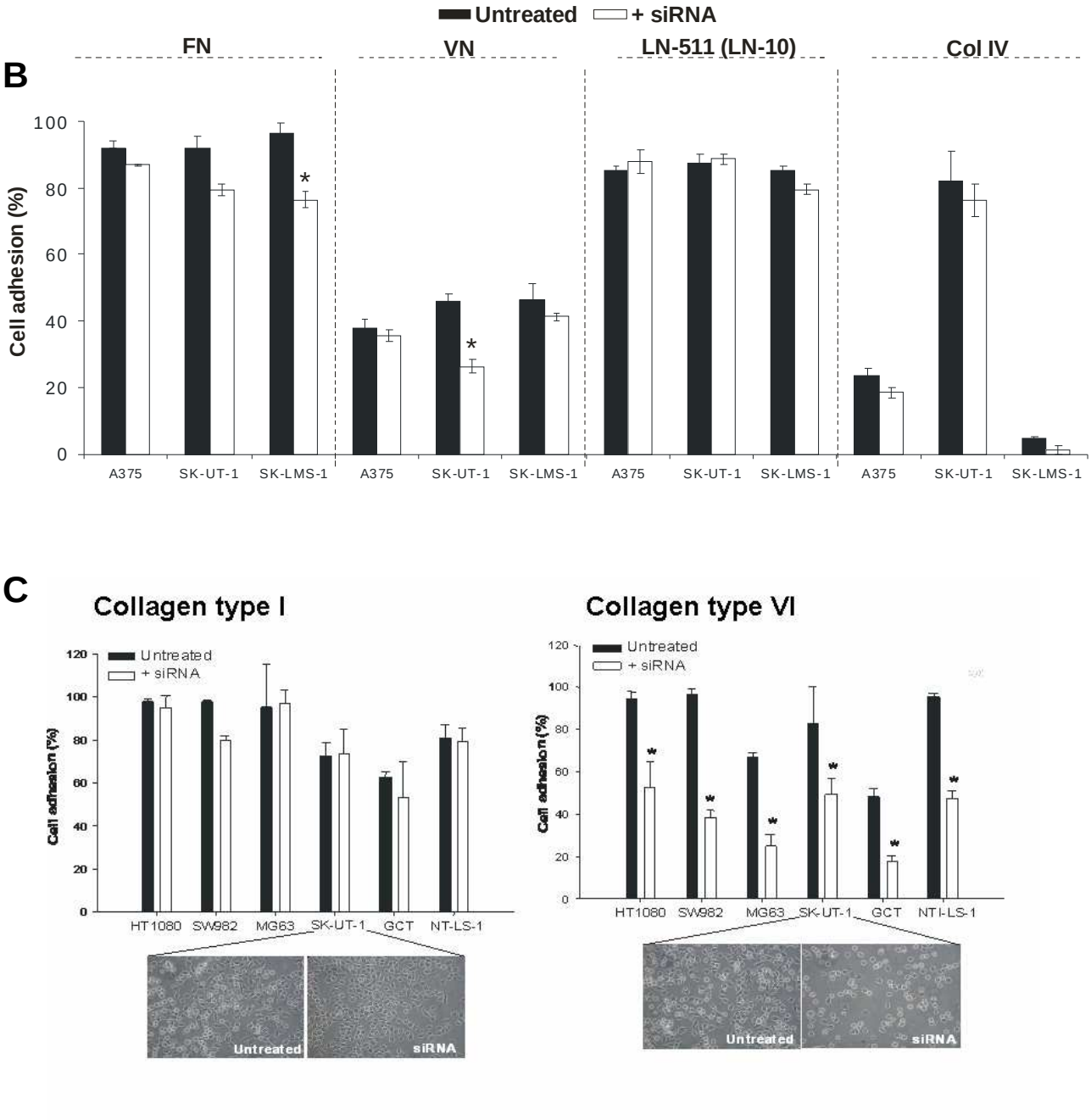


Figure 34. Cell adhesion after silencing of NG2. **A).** NG2 silenced cells were verified by flow cytometry. An exemplified for the synovial sarcoma cell line SW982. Largely comparable abrogation levels were obtained in the other sarcoma cell lines; **B)**. Comparing of adhesion of siRNA treated and untreated cells on different matrices quantified using CAFCA system; p<0,01 **C).** Quantification of CAFCA system. Images were captured using camera connected phase-contrast microscope (40x); p<0,01.

Comparison of adhesion of siRNA treated and untreated cells to collagen type VI underlined that NG2 abrogation significantly reduces both adhesion and spreading of the cells on this substrate and this effect seems rather specific for this collagen since little or no inhibition was seen on other representative ECM components.

Since the sarcoma cell lines used for this study were a mixed population of NG2 positive and NG2 negative cells, SK-UT-1 cells has immunosorted with MACS-based magnetic beads separation (Cattaruzza et al., 2009). Relative efficiency of the immunosorting

procedure and approximate yield of NG2+ was an average 18,7% for SK-UT-1 cells. CAFCA assay was performed for SK-UT-1 NG2+ and SK-UT-1 NG2- immunosorted cells on collagen type VI, fibronectin and collagen type I substrates (**Fig. 35**).

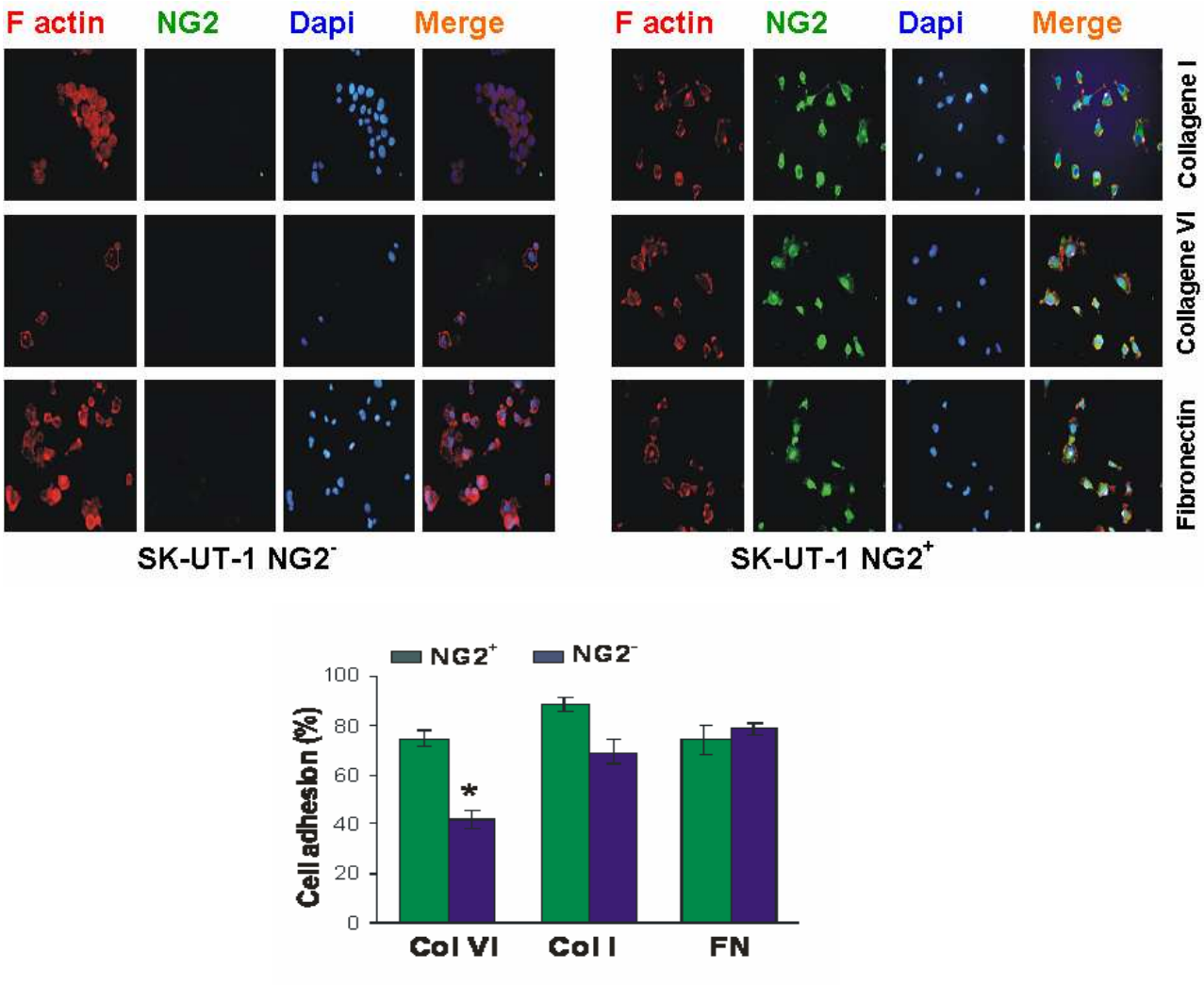


Figura 35. Adhesion of SK-UT-1 NG2+ and NG2- cells imnusorted. **A).** Representative views of attachment and spreading of $NG2^+$ and $NG2^-$ cells. Cells on the indicated substrates as revealed by phalloidin-Texas red staining. **B).** Corresponding quantitative analysis of cell adhesion to the same substrates; * p<0,01**.**

Comparing the adhesion of two subpopulations on collagen type VI has underlined different binding and spreading of NG2+ and NG2- cells on substrates. Whereas adhesion to collagen type I and fibronectin was not distinguishable.

3.12. NG2 involvement in the movement of sarcoma cells on collagen type VI substrate

Since NG2 influences the migration of tumour cells we tested migration of sarcoma cells with different surface levels of NG2 on different ECM substrates (**Fig. 36**).

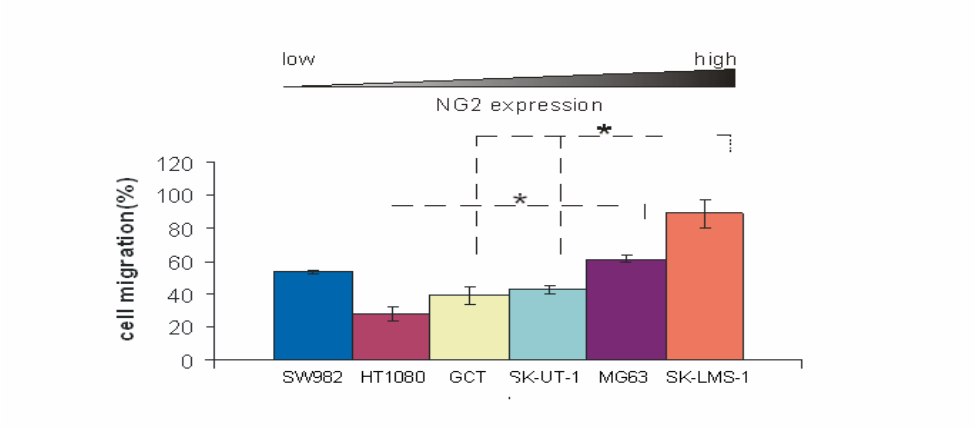


Figure 36. Relative levels of cell migration of different sarcoma cell lines exhibiting diverse constitutive levels of NG2 expression. In several pair-wise comparisons the levels migration of high NG2 expressing cells were found to be significantly higher than those of the low expressing ones (*, *p<0,001)*

It was interesting to note again sarcoma cells showed a migration ability in response to collagen type VI substrates that closely correlated with the endogenous levels of NG2 surface expression. In order to examine if NG2 was really involved in migration, sarcoma cells were NG2 silenced with siRNA. We have chosen to analysis the haptotactic migration of treated and untreated SK-LMS-1 cells because they had a high migration percentage and easily detectable. The migration of SK-LMS-1 silenced and untreated cells were monitored on collagen type IV, collagen type VI and collagen type I (as a control; **Fig 37**).

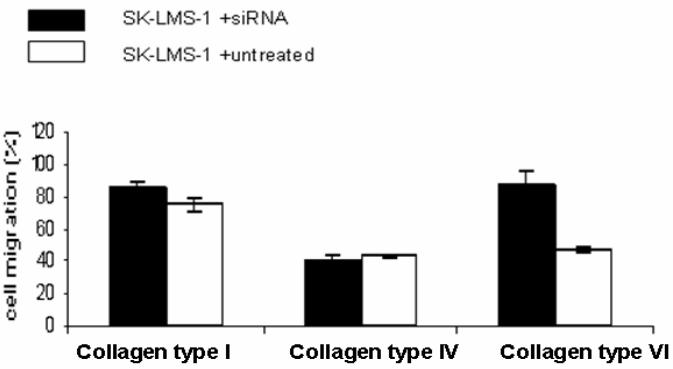


Figure 37. Haptotactic migration of treated siRNA and untreated cells on substrates. Selectively reduced haptotactic movement of RNA-mediated NG2 knockdown.

A 3-dimensional migration assay was performed by coating the transwell upper side with Matrigel. Collagene type VI was embedded in polymeric collagen type I substrates and Matrigel (**Fig. 38**).

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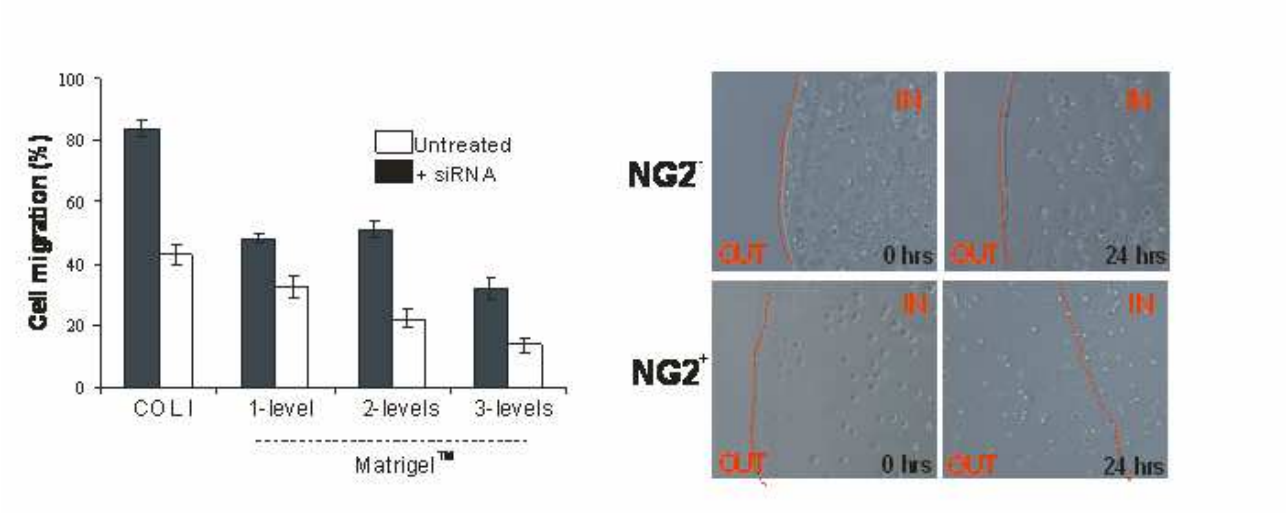

Figure 38. Reduced migration of sarcoma cells in response to Collagene VI after NG2 knockdown when examined in a 3D setting. Haptotatic migration was detected using SPCTRAfluor fluorometerI ($p<0,001$). Images were captured using camera connected to confocal microscopy

Also in this case RNAi-mediated NG2 silencing strongly compromised invasion of cells to move through collagene VI-containing Matrigel, in comparison to their counterpart.

We examined the interaction between sarcoma cells and different endothelial cells (HUVEC, HLEC, HCAEC and HIAEC). Transendothelial migration was performed using SK-UT-1 NG2+ and NG2- immunosorted. The kinetics of transmigration was examined in different times (0, 2, 5, 8, 24 and 30 hours; **Fig. 39**).

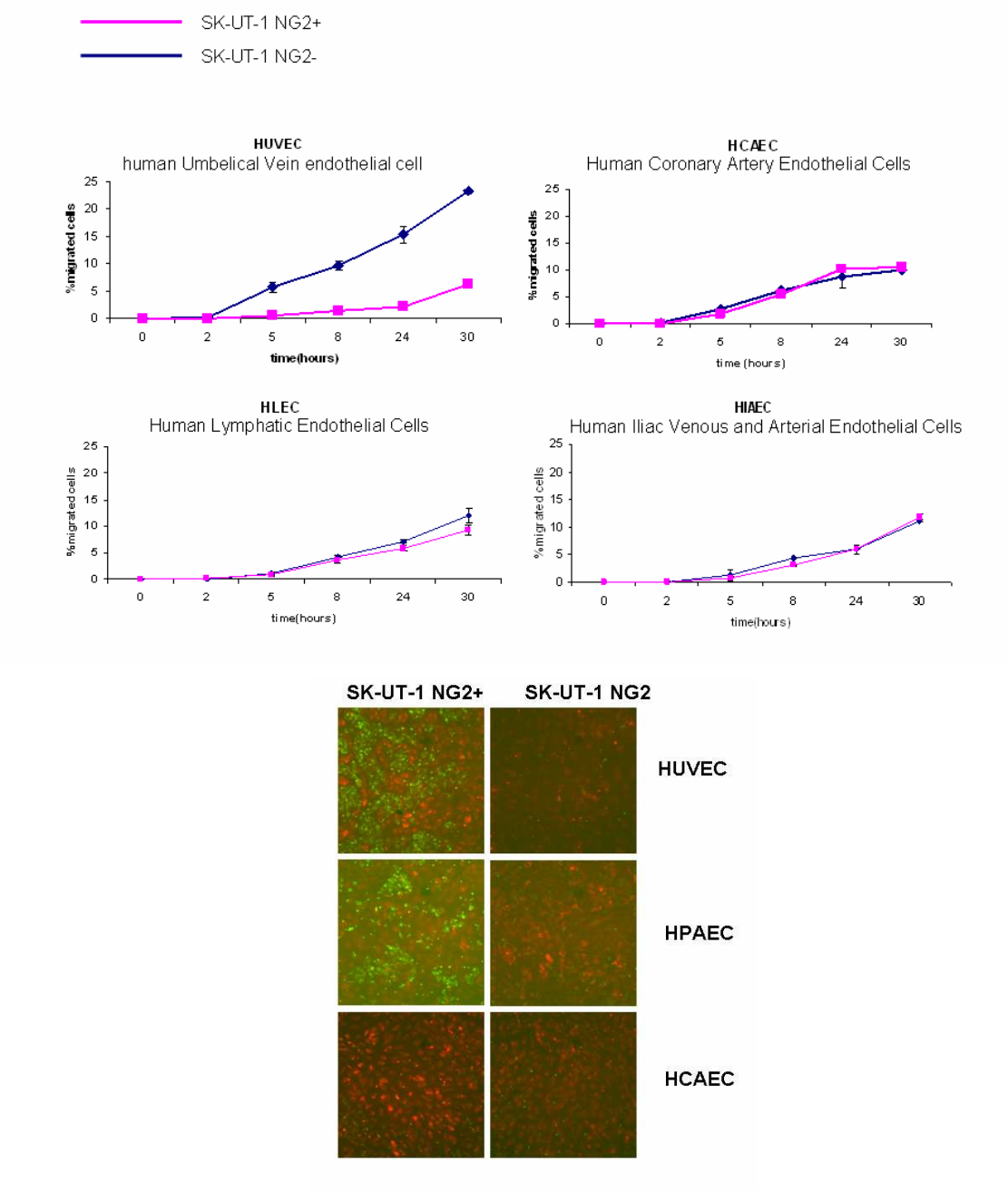

Figure 39. Transmigration assay**. A**). Kinetics of migration of two NG2 SK-UT-1 subpopulation; the assay was performed in duplicated; **B).** SK-UT-1 NG2+ and NG2- immunosorted cells were previously labelled with GreenCell Tracker. The endothelial monolayers were labelled with OrangeCellTracker..

The results showed a different transmigration behaviour of SK-UT-1 NG2+ and NG2- immunosorted cells only on HUVEC but not in others endothelial monolayer. It was interesting to observe that the percentage of two SK-UT-1 subpopulation migrates in the others endothelial monolayer less (about 10%) than SK-UT-1 NG2+ (about 25%) on HUVEC monolayer.

4. Discussion

Cell surface PGs are multifunctional molecules with roles in adhesion and growth factor signalling and are gradually emerging as key regulators of tumour progression. There are currently 11 membrane-bound PGs known in man and a certain number of them have been proposed to influence tumorigenesis by acting upon local tumour growth and/or by contributing to the metastatic processes. Increased or de novo induced expression of both secreted and cell surface-associated PGs may be a characteristic traits of invasive tumours solid. However paradoxically, these macromolecules often exhibited counteracting tumour growth-promoting and tumour progression-inhibiting functions depending on the tumour type in which they are mixepressed (Nurcombe et al., 1993; Cattaruzza et al., 2008). Nevertheless aberrant distribution of PGs observed in various tumours has incited a wealth of investigations on their potential diagnostic-prognostic, as well as therapeutic role. Several of these macromolecules are assuming important roles as prognostic factors and few are approaching the clinics. Given the dual tumour-promoting and tumour-suppressing role that cells surface PGs may play it is interesting to investigate whether different combinations of cells surface PGs actually synergize to promote tumour progression or act antagonistically to impede tumour growth and metastasis formation. The mode by which certain cell surface PGs may participate in the tumorigenic process has in some cases been extensively investigated (Fico et al., 2007; Sanderson et al.,2007; Munusue et., 2007; Williamson et. al, 2007) several have been found to exert a potential antineoplastic role by interfering with cell surface components regulating cell growth and maintenance of a differentiated phenotype. Since there is no information about surface PGs may affect the malignancy in sarcomas we have performed a preliminary screening for determining the surface PG profile in established cell lines and cells isolated from patients by qPCR and immunocitochemistry. This screening has allowed us to establish 24 surface PG pattern in sarcoma cells with an average of 5-8 PGs preserved on each cell type with the exception of 11 PGs expresses in SJRH30 rhabdomyosarcoma cells, 10 PGs detected in 143B and NTI-OS-5 osteosarcoma cells at the transcript level and only 1 surface PG expressed by NTI-LS-2 leyomiosarcoma cells. Literature data and our preliminary observations suggest that given combinations of surface PGs may diversely dictate the behaviour of cancer cells during distinct phases of tumour progression,. In fact our surface PG screening show how the same surface PG profile have been identified in cells belong to different hystotypes which could be in direct relation to the aggressive behaviour of the cells. Characteristically, in progressing tumours two patterns emerged: loss or neoexpression of surface PG depending on the initial expression pattern of the cell type of origin (Timar et al, 2002). Altered expression of surface PGs was detected in sarcomas respect with hMSC cells which could be the putative cell of origin of these tumours. In this study we aimed the asserting hypothesis that well-defined cell surface PG profiles may influence in specific way cancer cell motility, spreading and host microenvironment interactions. The relative expression of surface PGs in 143B osteosarcoma cells showed down regulation of all PGs. Till to day there are no notice about GPC6 implications either in cell-ECM

interactions or in behaviour motility of cells. For these reasons 143B cells were stable transfected for overexpressing GPC6 showed a modulation of relative expression of all surface PGs with changes of GPC1, 2, 3, 4, 5, 6 and SDC1 expression compared to control. Nevertheless, the GPC5 transcript level is highly up regulated in 143B overexpressing cells (about 670 fold) respect to control in which it is relatively absent. GPC6 overexpression induces morphological modification in 143B cells which are seen as changes in the organization of actin filaments containing protrusions similar to fillopodia/lamellipodia. Similar morphological changes have been observed in SDC4 overexpressing cells (Velleman et al., 2007). Moreover GPC-1 and SDC4 are able to regulate cell proliferation (Velleman et al., 2007) and could be correlated with a reduction of proliferation of 143B GPC6 overexpressing cells. The overexpression of GPC6 triggers with an increase of motility cells in monodimensional assay which could be in relationship with cytoskelatal reorganization revealed by immunoassaying. The effect of GPC6 on cell motility has been confirmed from Cell Migration Knowledgebase (CMKB; http://www.cellmigration.org) database where it is showed how MCF-10A cells GPC6 silenced migrate lower than untreated cells. Since either cell-cell interactions or cell-ECM interactions play a pivotal role in many of the phenomena of tumour progression we are going to examinee how cellular contacts with the ECM are mediated via membrane-bound proteoglycans (Silbert et al., 2003). HS chains have particular confidence to bind to collagen type III and -V, fibronectin, vitronectin and laminin-1. So we investigated 143B GPC6 overexpression ability to spread and to respond to these ECM molecules. In fact overexpression of GPC6 in 143B cells is linked to a reduction of behaviour adhesion on collagene III and also on uncoated substrates, greater adhesion ability on laminin-1 substrate than control cells. Moreover a greater spreading of 143B GPC6 overexpressing cells are observed on all ECM molecules with the exception of vitronectin where they have showed lower adhesion ability and lack of stress fibres. Nevertheless 143B GPC6 overexpressing cells has a greater invasion ability in response to collagene type III than vector control cells and this is unchanged in response to collagene type V and reduced further in response to vitronectin. In particular the spreading ability and the lower invasion ability of the transduced cell line by overexpressing GPC6 on different ECM molecules could be correlated by surface PG profile modulation. SDC4 overexpression allowed cell spreading on vitronectin with formation of focal adhesion (Echtermeyer et al., 1999) explaining the adhesive behaviour of 143B GPC6 overexpressing and control cells on this substrate. Nevertheless cell-ECM interactions are mediated also by integrins that transmit signals from the ECM into the cells and vice versa [90-91] so it will be interesting to observe the existence of collaboration between surface PGs, in particular HSPGs and integrins (Juliano et al., 1993; Woodhouse et al., 1997). Preliminary observation reveals a partial disappearance of GPC6 from surface of 143B GPC6 overexpressing cells over time. GPI-anchored proteins are usually associated with sphingolipid- and cholesterol-rich plasma membrane domains. Such enriched domains may exist either as small phase-separated "rafts" or, when associated with caveolin-1 (Cav-1), form flask-shaped

plasmalemmal invaginations called caveolae, which are involved in signal transduction and special forms of non-clathrin-dependent endocytosis mediated by Cav-1-containing endosomes, also called caveosomes (Drab et al., 2001). GPC1 has been associated with lipid rafts, caveolae, caveosomes, or endosomes transporting cargo from the plasma membrane to the Golgi. Using anti-Rab5 as a marker for late endosomes (Barbero et al, 2002), colocalization with GPC6 are seen in small vesicles in cytoplasm. This observation will be validated with more detailed analysis using confocal laser microscope.

To dissect the molecular mechanisms that may govern the neoplastic behaviour of sarcoma cells with different relative surface levels of the above glypicans we will select the model cells that through the combination of our *in vitro* and *in vivo* assays have emerged as those with the strongest and poorest aggressive phenotype and on these we are performed combined antibody array- and immunoblotting-based phosphorylation analyses (adopting platforms provided custom by Kinexus Bioinformatic Corporation, Canada).

The idea that NG2-positive cells may be particularly aggressive was sustained by comparing the *in vivo* behaviour of NG2-positive and NG2-negative subsets implanted into immunodeficient mice: the former sarcoma cells grew into a defined mass more rapidly and more extensively than the latter ones (Cattaruzza et al., 2009). This observation is consistent with our *in vitro* findings showing sustained anchorage-independent growth of $NG2^+$ cells and recalls the potential influence of the PG in the control of cell survival and proliferation. In fact, in a separate, recent study from our group we demonstrate that NG2 profoundly affects mitogenic responses of sarcoma cells to FGF family members (Cattaruzza et al., 2008). However, since some of the $NG2^+$ cells investigated here failed to engraft and grow in nude mice, the pro-tumorigenic role of NG2 may not be restricted to a mere control of cell proliferation. Conversely, we hypothesize that high surface levels of NG2 may confer a more malignant behaviour in sarcoma cells by controlling multiple facets of the tumourigenic process. Analyses of the in situ distribution of the primary ECM ligand of NG2 revealed that up regulation of the NG2 in neoplastic cells of primary sarcoma lesions was accompanied by a corresponding augmented host synthesis and deposition of the collagen within the lesion (Cattaruzza et al, 2009). It seems that an overabundance of the collagen in the host microenvironment did not seem to constitute an effective prognostic/predictive factor. A restricted expression of Collagen type VI in the tumour stroma was also consistent with previous observations indicating that mesenchymal tumours may loose the expression of the collagen upon neoplastic transformation (Schenker and Trueb, 1998). Our present data support this notion and show that only a few of the sarcoma cell lines examined in this study displayed detectable levels of Collagene VI transcription and translation. Anyway high levels of Collagen type VI deposition in NG2-rich metastatic lesions suggested to us that the previously reported NG2-collagene type VI molecular interaction could play a crucial role in tumour cell-host microenvironment interplays, not

only during local growth but also during the dissemination and homing phases to reach the putative metastatic sites. We examinated the interactions of NG2- and NG2+ sarcoma cells with collagen type VI and other ECM molecules. To support this hypothesis sarcoma cells were NG2 abrogated by RNAi and immunosorted for NG2 expression were found to exhibited a rather elective impaired ability to adhere and migrate on purified collagene type VI. These findings confirmed that the NG2 was able of mediating tumour cell adhesion and migration on Collagene type VI substrates with a direct interaction with the collagen and largely precluded that the NG2 acted solely as a modulator of the function of collagen-binding integrins (in our case of the α2β1 integrin; Chatterjee et al., 2008). Furthermore, these data strongly supported the notion that enhanced growth and metastatic behaviour of NG2-expressing sarcoma and melanoma cells could be directly mediated by a local interaction with collagen type VI present within the tissues/organs infiltrated by metastasizing cells or overproduced and secreted within the tumour lesions by stroma-forming cells. These preliminary linear findings corroborated the role of the NG2-ollagene type VI interplay in the potential regulation of tumour cell spread. NG2 may represent a unique, independent prognostic factor in several types of soft-tissue sarcomas in which its relative levels of expression in primary lesions are additionally capable of predicting the future occurrence of post-surgical metastases. Further multicenter studies are now in progress to establish whether NG2 may be proposed in the routine clinical practice and in which sarcoma types, else than those examined herein, it may be used as a metastasis predicting factor.

5. Acknowledgements

L'arte e la scienza sono libere e libero ne è l'insegnamento.

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