UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Fisiopatologia Sistemica

Ciclo XX

DEFINING THE CELL SURFACE PROTEOGLYCAN METASTATIC SIGNATURE OF CANCER CELLS: ROLE OF GLYPICAN-5 IN THE CONTROL OF CELL MOTILITY

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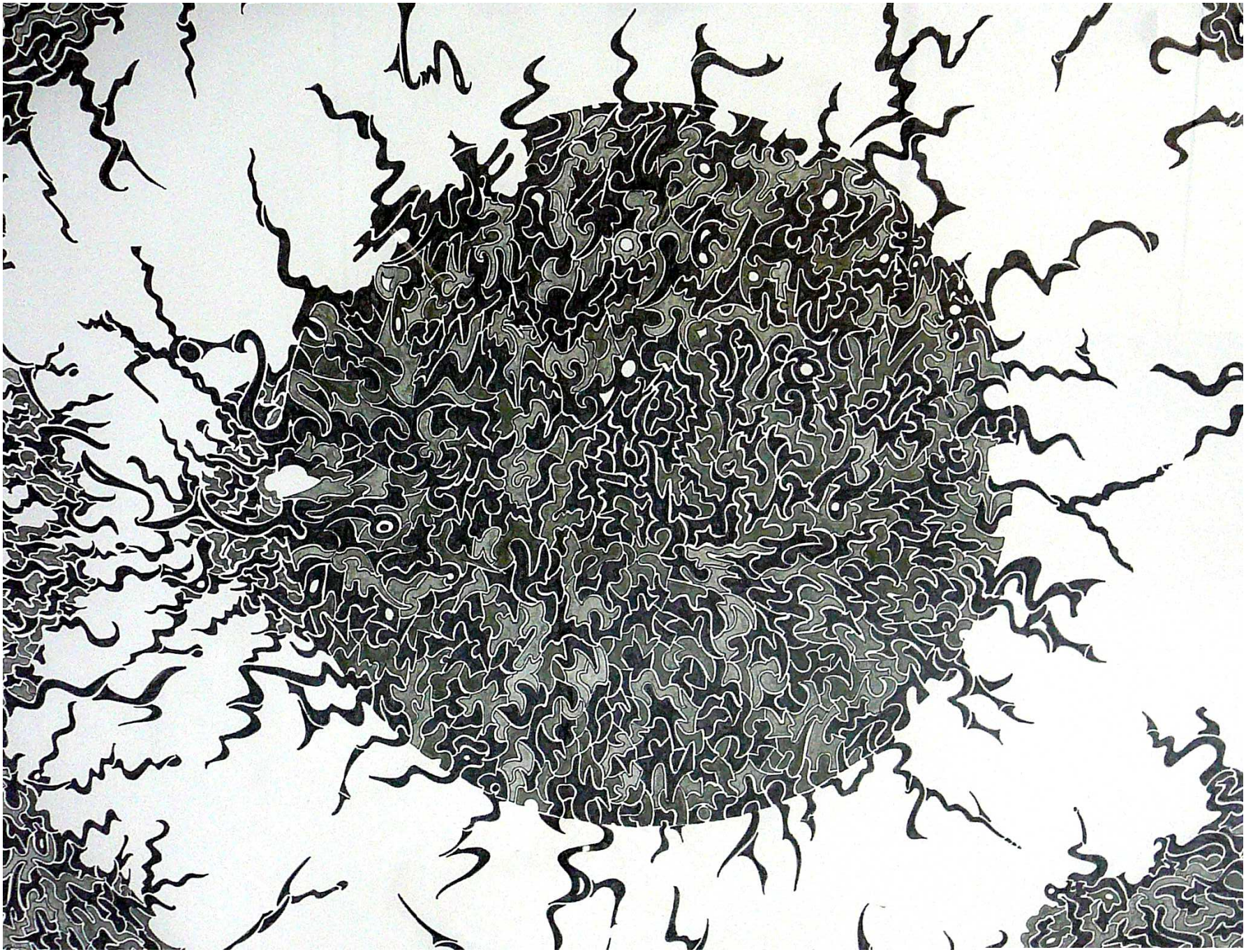


Tra caos e cosmo, 1989

a mia Madre e mio Padre

The ultimate measure of a man is not where he stands in moments of comfort and convenience but where he stands at times of challenge and controversy
(la qualità di un uomo non si vede nei momenti di benessere e di comodità ma come si pone di fronte alle sfide ed alle controversie)

The worth of an individual does not lie in the measure of his intellect, his racial origin, or his social position
(il valore di un individuo non risiede nella misura del suo intelletto, nelle sue origini o nella sua posizione sociale)

Martin Luther King Jr.

a Davide e Gaia

«Lungo il sentiero ripido e pietroso, ho incontrato una piccola bambina che portava sulla schiena il suo piccolo fratello. "Bambina mia - le ho detto - stai portando un pesante fardello!". Lei mi ha guardato e ha detto: "Non è un fardello, signore, è mio fratello!"»

Racconto di un missionario, Perù

INDEX

SUMMARY

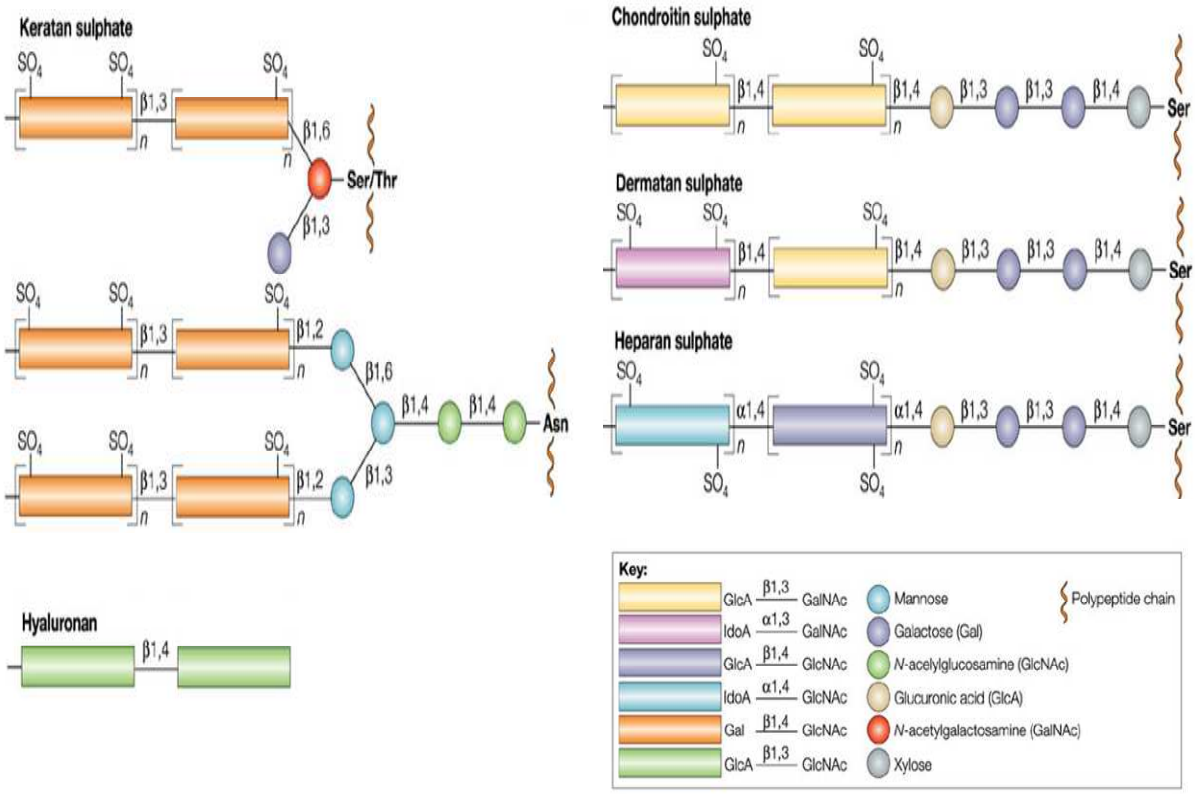
Surveying literature data, what emerges is that cellular function and phenotype are highly influenced by cell surface proteoglycans (PGs), present at the cell-tissue-organ interface where they are thought to have crucial regulatory roles in cellular control and in coordinating and directing appropriate response to multiple ligands in normal physiological processes and pathological conditions, including the processes of tumor onset and progression. The complexity of PG surface pattern in tumour cells, especially that provided by glypicans, syndecans and NG2, enables them to modulate directly or indirectly several aspects of the tumour cell phenotype, including growth kinetics, invasiveness and metastatic potential. Recent evidence further highlights the fact that cancer cells, as part of the transformation process, alter their cell surface PG profile, but also alters the fine structure of a given proteoglycans and the tumorigenic function of the 11 primary PGs, including syndecans-1-4, glypicans-1-6 and NG2, is only in part resolved for some of these PGs individually, but is obscure when considering these molecules cumulatively. In this context, we have focused our research on trying to understand how a given cell surface PG pattern may be involved in tumour growth and metastasis in sarcomas and whether they could represent new potential prognostic/predictive markers in defined class of patients with oral squamous cell carcinomas (OSCC). We have approached this problem defining systematically the constitutive pattern of cell surface PG expression in a large panel of sarcoma cells and OSCC biopsies combining analyses at the transcript and immunochemical level and by creating 143B osteosarcoma cells over-expressing glypican-5. This engineered cellular model was then assayed for its adhesion and migratory abilities in response to isolated molecules of the extracellular matrix *in vitro*. Misexpression of glypican-5 on defined constitutive PG backgrounds modulates the in vitro behavior of the cells. The outcome of this project could provide the preliminary evidence that defined surface PGs pattern could differentially control cancer cells behavior and could be a new perspective in the understanding how these molecules regulate different aspects of cancer biology.

1. INTRODUCTION

1.1 PROTEOGLYCANS

Proteoglycans (PGs) represent a special class of glycoproteins that are heavily glycosylated. They consist of a core protein with one or more covalently attached glycosaminoglycan (GAG) chain(s). These glycosaminoglycan chains are long, linear carbohydrate polymers that are negatively charged under physiological conditions, due to the occurrence of sulfate and uronic acid groups. The biosynthesis of PGs, in addition to the ordinary biosynthetic processes of O- and N-linked oligosaccharide components as in other glycoproteins, further requires biosynthesis of GAG in the Golgi apparatus, which can be considered to be a most complex biosynthetic process for complex carbohydrates. It requires a number of glycosyltransferases and sulfotransferases and involves multiple sub regions of the Golgi apparatus, which poses a great challenge for researchers in elucidating the organization and regulatory mechanisms involved in PG biosynthesis. PGs have been originally classified according to the nature of their glycosaminoglycan chains (Figure 1): chondroitin sulfate, dermatan sulfate, heparin/heparan sulfate, keratan sulfate. Proteoglycans have also been subdivided according to their position in relation to the cell and by size. Extracellular PGs include large PGs of the hyalectan family (e.g. aggrecan, versican), basement membrane PGs (perlecan, agrin, bamacan) and small leucine-rich proteoglycans (SLRP). Several collagen types (IX, XII, XIV, and XVIII) have been shown to carry GAG chains and may therefore be considered “pseudo-PGs”. Several extracellular PGs do not belong to any family, they are often presented as ‘part-time’ or ‘facultative’ PGs (CSF-1, M-CSF, claustrin). The non-classified PGs include thrombomodulin and several neural PGs: neuroglycan, phosphacan, brevican[1,2]. Hematopoietic cells also contain a secretory vesicle proteoglycan known as serglycin[1]. Several excellent reviews describe the properties and structural-functional peculiarities of PGs[3,4,5,6,7,8]. The heterogeneity of GAGs and the existence of more than 50 genes coding for PGs give a plethora of molecular properties for these macromolecules. Often, the property of an isolated GAG chain is contradictory to those of the same chain attached to the protein core of PGs[9]. Individual functions of proteoglycans can be attributed to either the protein core or the attached GAG chain[10,11]. They hold a large number of water molecules in their molecular domain and occupy enormous hydrodynamic space in

solution. Another remarkable property of the glycosaminoglycans, which is particularly significant in heparan sulfate and heparin, is their capability to specifically interact with a number of important growth factors and functional proteins.



From: Kleene, R., Schachner, M. Nature Reviews Neuroscience 5, 195-208 (March 2004)

Figure 1: Schematic structure of glycosaminoglycan and proteoglycan. Hyaluronian is not a PG linked to GAG and is free-standing thin filaments assembled in the ECM and around the cells.

Proteoglycans are a major component of the animal extracellular matrix, the "filler" substance existing between cells in an organism. Here they form large complexes, with other proteoglycans by binding filaments and by linking to matrix proteins (such as Collagen). There is evidence that they can affect the activity and stability of proteins and signalling molecules within the matrix. They are also involved in binding cations (such as sodium, potassium and calcium) and water, and may regulate displacement of molecules through the matrix (Table 1). The HS chains at the cell surface are mostly attached to

syndecan and glypican core proteins. These core proteins determine the proximity of the HS chains to the cell surface, when, where, and to what extent the HS chains are expressed and the rate and mechanism of HS turnover[12]. Heparan sulphate-bearing proteoglycans (HSPGs) are divided into three subfamilies: the membrane-spanning proteoglycans (syndecans, betaglycan and cell membrane hyalectans as CD44), the glycophosphatidyl-inositol (GPI)-linked proteoglycans (glypicans) and the secreted extracellular matrix (ECM) proteoglycans. The HS chains are assembled on core protein by enzymes in the Golgi, using nucleotide sugar imported from the cytoplasm. There is great structure heterogeneity in term of chain length and size (examples of large proteoglycans are aggrecan, the major proteoglycan in cartilage, and versican, present in many adult tissues including blood vessels and skin. The small leucine rich repeat proteoglycans (SLRPs) include decorin, biglycan, fibromodulin and lumican), the modification on the chains (deacetylation, sulphation and epimerization) and spacing of the modified tracts[1]. Because of their negative charge, the HS chains bind a very wide type of proteins, including the members of the fibroblast growth factor (FGF) family and their receptor tyrosine kinase, transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), Wnt proteins, chemokines and interleukins, as well as enzyme inhibitors, lipases an apolipoproteins and ECM an plasma proteins. Many functions of HSPGs depend on interaction of the core protein. For example, HSPGs at the plasma membrane can transfer spatial information about a cell's environment and either activate adhesion mechanisms or enhance cell motility. Often, signaling through HSPGs in this context occurs in collaboration with other cell-surface receptor (e.g. integrins) to facilitate cell attachment, spreading and motility[1] (Table 2).

Table 1: Some of the putative molecular interactions of exhibited by the side chains of PGs

GAG	ECM components	Signalling molecules	Cell adhesion molécules	Other molecules
Heparin heparan sulphate	Fibronectin Fibrin Collagen types I, III, V Laminin-1 Pleiotropin Tenascins-C, -R Thrombospondin Vitronectin Fibrillin-1 Fibulin-2 Slit-1,-2	FGF family members 1-24 (?) HB-EGF HGF/SF IGF-I, IGF-II Wnt1, Wnt3a (other Wnts ?) Chordin Noggin Slit/Robo Interferon γ PDGF SDGF VEGF TGF β 1,2 Midkine Pleiotropin Angiopoietin-3 GM-CSF IL-8 IP-10 MCP-1, -4 RANTES TNFα.β	N-CAM L-selectin P-selectin MAC-1 PECAM-1	Acetyl cholinesterase Thrombin Lipoprotein Lipases Heparin Cofactor II Neutrophil elastase Plasminogen-Activator Inhibitor MMP7 TPA ApoB ApoE Amyloid-precursor protein Amyloid-precursor-like protein-2 Cathepsin-X Viral coat proteins (*Tat*, papilloma virus, cytomegalovirus) Microbes and microbial products
Chondroitin sulphate	HA Tenascins-C, -R CMP Fibulin-1, -2 Fibronectin Fibrillin-1 Amphoterin Collagen types V, VI	HB-GAM/PTN FGF-2, -16, -18 PDGF-AA Angiostatin Midkine Pleiotropin Amphoterin HB-EGF VEGF SLC IP-10 SDF-1ß	N-CAM Integrins ($\alpha_5\beta_1/\alpha_4\beta_1$) CD44	PDGFRα FGF receptor-1, -3 MT3-MMP GRIP-1 LDL Glycolipids
Dermatan sulphate	Collagen types I, II, VI, XII Matrilin 1 Tropoelastin Matrix Glycoprotein-1 Fibronectin Tenascin-X	FGF-1, -2, -7 HGF/SF TGFβ PF-4 SLC IP-10 SDF-1ß RANTES	ICAM-1 L-selectin P-selectin, CD44	Heparin Cofactor II Thrombin Activated Protein C Protein C inhibitor LDL and HDL
Keratin sulphate(s)	Collagens			

Table 2: Predicted functional traits of primary HSPGs involved in the regulation of cell movement[1]

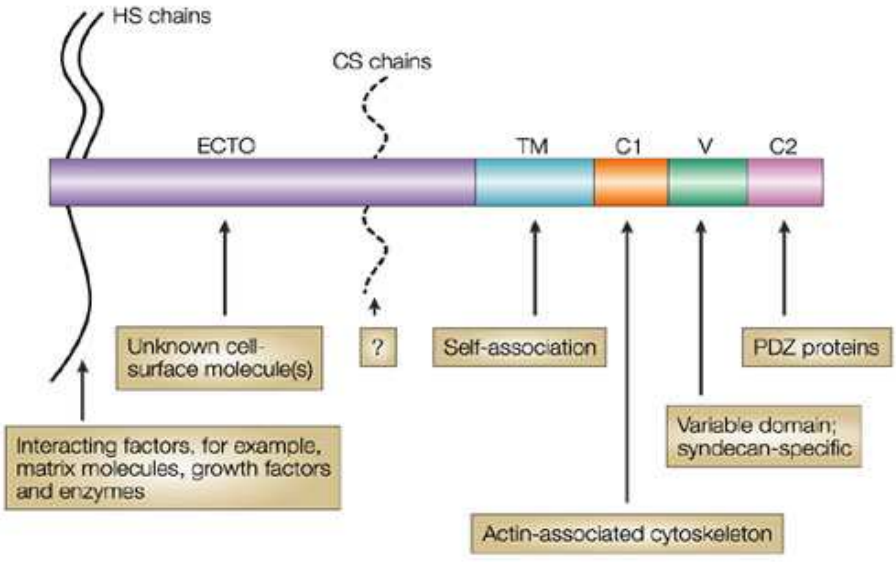
HSPGs	Non permissive directional cue[2]	ECM linker of direction-promoting cues[3]	Cryptic motility-promoting factor[4]	Shedded motility-inhibitor[5]	Cell surface interactor[6]	Enhancer of signal transduction[7]	Cell-ECM co-receptor[8]
CD44				(X)	X	X	X
perlecan	X	X	X				
syndecan-1				X	X	X	X
syndecan-2				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		

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

In addition to their structural domains, HS core proteins are all posttranslationally regulated; particularly all HS core proteins can be shed from the cell surface. In many cases, shedding converts the HSPG from an activator to a potent inhibitor[13,14]. Shedding of syndecan itself is tightly regulated[15] and can be used by pathogens as a means of cellular entry[16]. Syndecans shedding is stimulated by epithelial wounding, where release from the cell surface is mediated by matrix metalloproteinases. In mammalian physiology, protein-HSPGs interactions promote activities specific to each organ system to regulate metabolism, transport, information transfer, support and regulation at the systemic level as well as cellular level[1,17,18]. HSPGs are also gradually emerging as key regulators of tumour progression and several of them are assuming important roles as prognostic factors.

1.2 SYNDECAN FAMILY

Syndecans represent a four-member subfamily of highly conserved type I transmembrane heparan sulfate PGs and are expressed on virtually all cell types throughout development and adulthood. The syndecans' core proteins show a high degree of conservation in the cytoplasmic domain (ca. 30 amino acids) and transmembrane domain and this suggests that 4 genes arose from an ancestral common gene and sequent divergent evolution and duplication: syndecan-1 with syndecan-3 and syndecan-2 with syndecan-4. The cytoplasmic domain is partitioned in three regions: C1 (juxtamembrane region) and C2 (C-terminal region) conserved, V (intervening region) variable and different for each syndecan, but conserved across species. C2 region has the amino acid sequence EFYA which binds PDZ (PSD-95, Dlg, ZO-1) domains present in specific proteins, like Ca^{++}/Calmodulin-dependent serin protein Kinase (CASK)[19] (Figure 2). This binding allows assembling protein complexes on the inner surface of the plasma membrane and to link membrane components to the actin microfilament of the cytoskeleton.



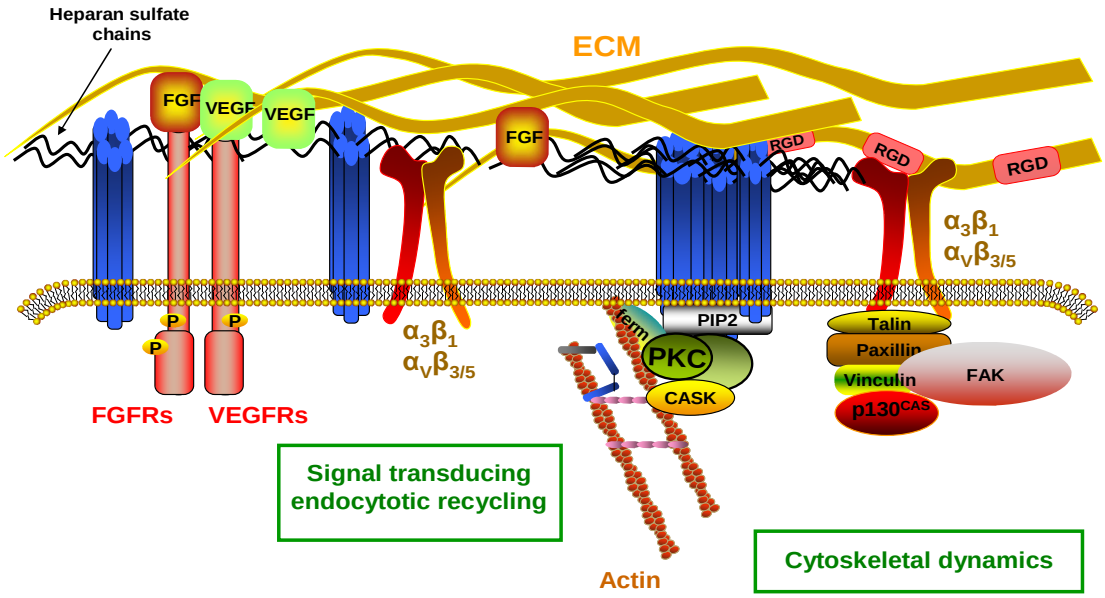
From: Couchman, J.R. Nature Reviews Molecular Cell Biology, 4: 926-938 (Dec 2003)

Figure 2: A schematic view of a syndecan, showing the core protein and glycosaminoglycan chains. Potential interactions, where known, are indicated. CS, chondroitin sulphate; ECTO, ectodomain; HS, heparan sulphate; TM, transmembrane domain; C1 and C2 refer to conserved regions 1 and 2, and V refers to the variable region, of the cytoplasmic domain

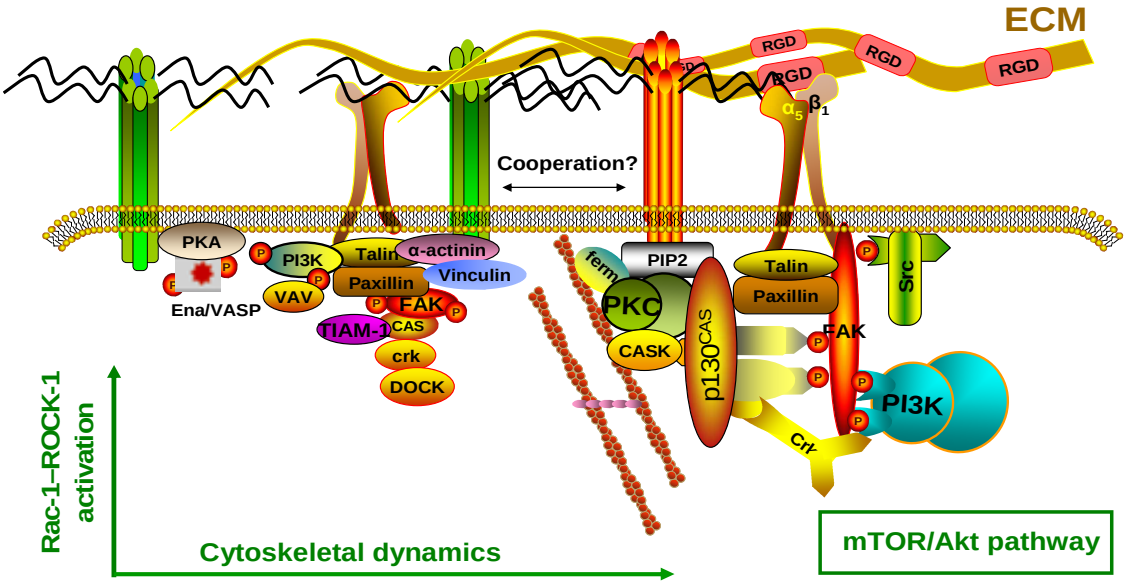
Each member of the family has a distinct temporal-spatial pattern of expression. Syndecan-1 is expressed predominantly in epithelial at the basolateral surface of cells and mesenchymal tissue and in *in vitro* malignant transformation syndecan expression is associated with maintenance of epithelial morphology, anchorage-dependent growth and inhibition of invasiveness; down-regulation in syndecan-1 expression shows a cell transformation from epithelial to mesenchymal and increased invasion [20,21]. In contrast to the general notion that the syndecan may be inhibitor of tumorigenensis, syndecan-1 shows a dual role because its enhanced expression has also been observed in gastric[22] and breast[23] carcinomas and this over-expression correlates with tumor aggressiveness and poor clinical prognosis. Syndecan-2, originally described as fibroglycan on fibroblast where it has been implicated in matrix assembly[24,25], also has important functions in nervous system; is expressed predominantly in embryonic development and recently it is suggest its role in regulation of TGF-β signaling[26]; syndecan-2 has a second role in adhesion and proliferation of colon carcinoma cells where is over-expressed[27]. Syndecan-3 plays an important role in regulation of skeletal muscle differentiation and development; also appears to be involved in regulation of Hh signaling and in mammalian one member of this family, indian Hh (iHh) is spatial-temporal regulated by syndecan-3[26]. Syndecan-3 is also found in the nervous system, where binds heparin-binding growth associated molecule (HB-GAM) in outgrowing axonal tracts of the developing thalamocortical pathway of the rat[25,28]. Syndecan-4 is the most studied member of the syndecan family and has a various activities, including modulation of FGF2 signaling, regulation of cell migration via β_2 integrin, control of adhesion via cytoskeletal modifications and its plasma membrane localization and participation in FGF2 endocytosis underlines its eventual relationship to lipid rafts, plasma membrane domain that provide internalization of membrane components[29]; another role of syndecan-4 involves regulation of fibronectin signaling and matrix contraction together with tenascin-C[30]. Up-regulation of syndecan-4 has been noted in hepatocellular carcinomas and malignant mesotheliomas[31] and such over-expression may correlate with increased tumor cell proliferation[32]. Furthermore, syndecans are shedded from cell surface via cleavage of their ectodomain; this process allows that free ectodomain play as a soluble regulatory macromolecules role[33]. Summarizing, the core proteins of syndecans

are involved in many signaling function that translate information to the cytosol from extracellular environment trough their heparan sulfate chains; nevertheless these informations, the mechanism which cooperate with integrin become unknown (Figure 3).

SYNDECAN-1



SYNDECAN-2/SYNDECAN-4



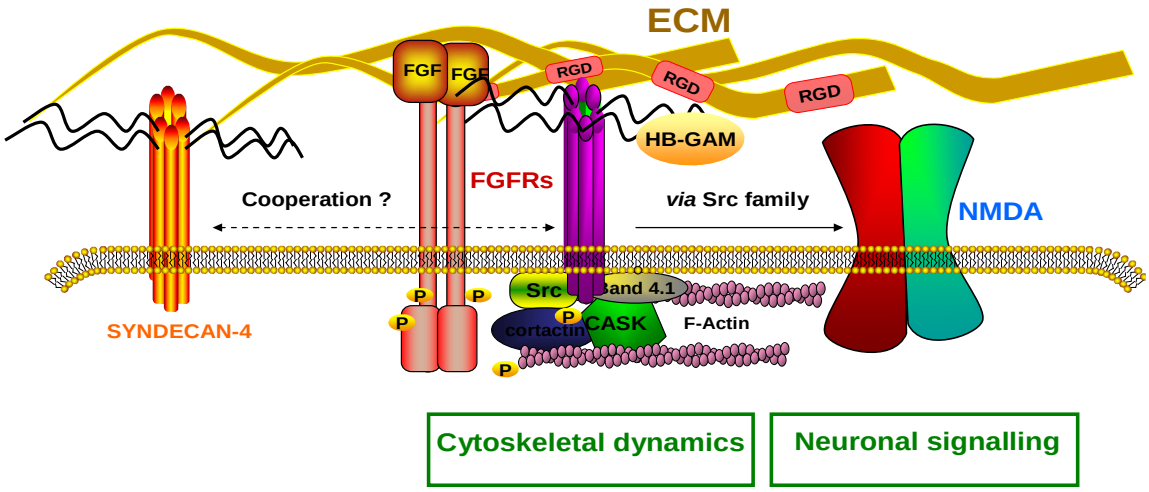
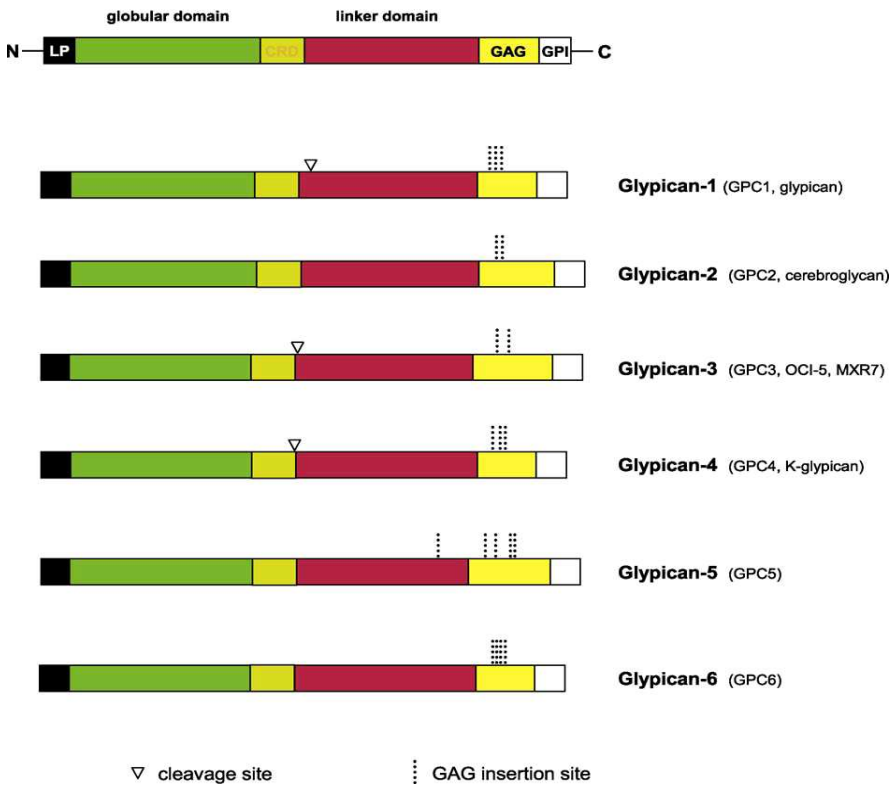


Figure 3: Schematic overview of the putative molecular functions of syndecans; they may participate in growth factors/cytokine signalling events through their HS chains and may additionally link to the same ECM components. Either individually or through a putative cooperation, syndecans strongly impact on cytoskeletal dynamics through phosphorylation events taken place in the cytoplasmic tail.

1.3 GLYPICAN FAMILY

The name glypican identifies a family of HSPGs that are linked to the cell surface of the plasma membrane through a covalent glycosyl-phosphatidylinositol (GPI) linkage[34,35]. Six glypicans have been identified in mammals so far (GPC1 to GPC6)[36,37,38,39,40]. The size of core protein of glypicans is similar (60 to 70 KDa) and they all contain an N-terminal secretory signal peptide and a hydrophobic domain in the C-terminal region required for the insertion of the GPI anchor.



From: Song and Filmus, Biochimica et Biophysica Acta, 1573 (2002), 241-246

Figure 4: General schematic structure of glypican core proteins.

Although the degree of amino acid homology between most glypican is moderate, the localization of 14 cysteine residues which are believed to form intramolecular disulfide bonds, giving all glypicans a conserved three-dimensional structure[41,42,43]. In addition to the GAG-link region, the mature core proteins have other two structural domains: the linker domain and an N-terminal globular domain identificated for an internal cleavage site in GPC1, GPC3, GPC4 and GPC5[36,44,45,46,47]; this reported cleavage site map in the same region between globular domain and linker domain and the significance of this proteolysis and the enzyme responsible is unknown. It's to point out that the 30-40 KDa cleavage product that is generated from the N-terminal of the protein remains attached to its C-terminal half through some disulfide bridges, as it is generally undetectable during Western blotting without reducing condition[36]. Another characteristic shared by all glypicans is the localization of the insertion sites for the HS chains, which seems to be

restricted to the last 50 amino acids in the C-terminus, place the chains close to the cell membranes[34,42]. Although there are vary degrees of glycosylation, all mature glypican protein cores are plus/minus 500 amino acids. The degree of amino acids homology between the glypican family tend to show the separation in two major group. The first group including glypican-1, gypican-2, glypican-4 and glypican-6 with 35%-63% sequence similarity; the second is comprised of glypican-3 and glypian-5, which are 54% similar[37], whereas the homology between of the two groups is only 17%-25%. In addition to the similarities in protein sequences, one of the typical element is the GPI anchor, which significance has not been clearly defined (Figure 5).

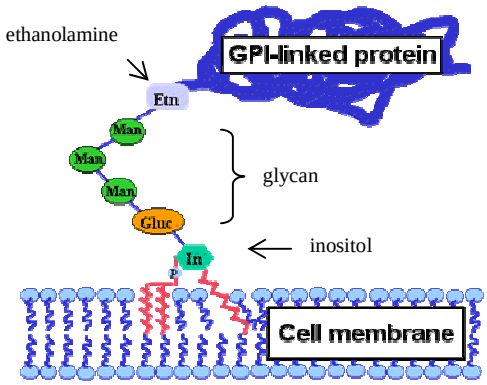


Figure 5**:** Structure of GPI anchor (From: www.hmds.org.uk)

There are some hypothesis about that: first is that the lipid-anchor targeting the glypican in specific areas of the cell membrane called "raft"[48,49] involved in Ca^{++} influx, the second possible role is their function in the release of the proteins in the extracellular environment by shedding, and the third hypothesis concern their recycling to the cell surface via endocytosis[50] regulating in this way receptor-mediated endocytosis and the uptake of molecules from extracellular environment. Particularly, the lipid anchor can be removed by proteases or lipases leading to shedding of glypican from the plasma membrane to generate either soluble or glypicans form associated with low-density particles. Recent studies unveil the cell-autonomous functions of glypican released that can be transported across tissue and elicit their function in a broader field of cells[51,52]. Recently, it was been demonstrate that a protein called Notum, an alpha/beta-hydrolase cleaves GPI anchor protein in mammalian and this process is direct correlate with

signaling of Wnt[53], where in *Drosophila* Notum was identificated as a novel inhibitor of Wnt signalling by modifying the HS chains of *Drosophila* glypicans[54,55], but remains unclear whether Notum is able to cleave glypicans from mammalian cells[56]. In general, glypicans are expressed predominantly during development[41]. In most cases where detailed studies have been performed, expression levels have been shown to change in a stage- and tissue-specific manner, suggesting that glypicans are involved in morphogenesis[38,43,57,58]. Glypicans appears to be expressed in spatial-temporal manner within developing tissue also through the passage from embryonic to adult (Table 3).

Table 3: Differential expression of glypicans during mammalian development

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

From: Jorge Filmus. Glycobiology 2001 11: 19R-23R

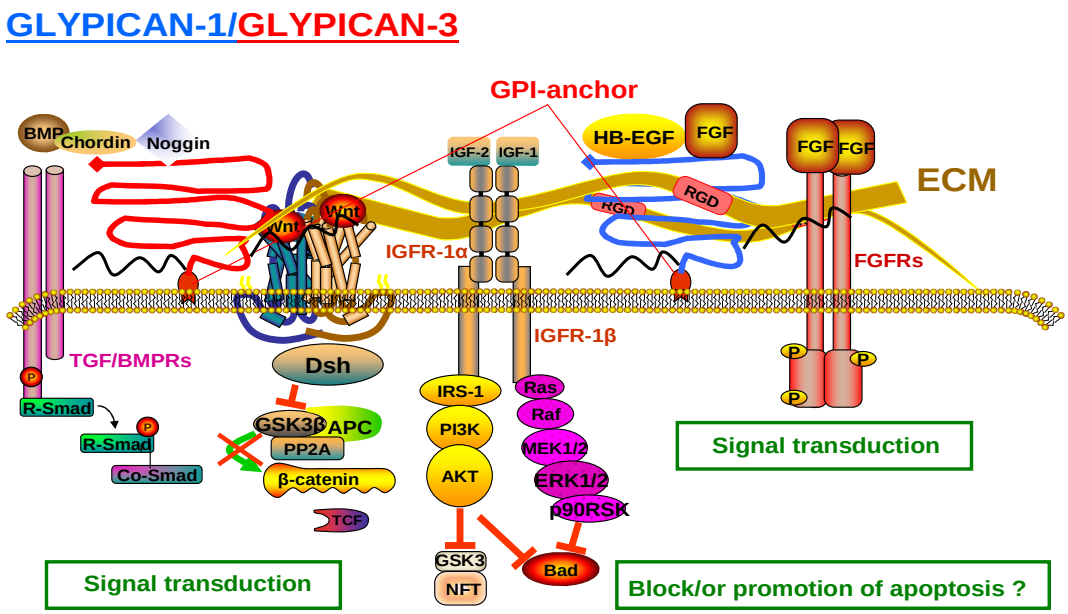
The role that they have in development embryonic and at last in tumour development is still unknown for the most of the glypicans, with the exception of glypican-3, for which there is a particular interest because is direct involved in Simpson-Golabi-Bhemel Syndrome[59] and for its high expression during mammalian development in most tissue except for the nervous system[60] (whereas in adult GPC3 expression is detected in very limited number of tissue) (Table 4).

Table 4: Clinical applications of glypicans in oncology

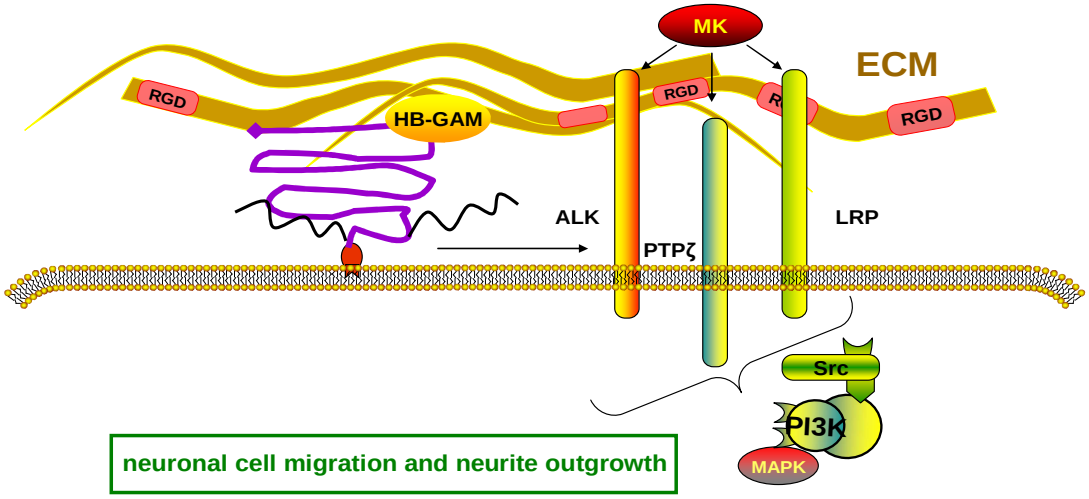
PG	Diagnostic	Predictive/prognostic
glypican-1	?	Pancreatic carcinoma
glypican-2	?	
glypican-3	Hepatocarcinoma (Melanoma)	Hepatocarcinoma
glypican-4	?	?
glypican-5	?	?
glypican-6	?	?

While studies on ovarian cancer cell lines, mesotheliomas, and breast tumors have demonstrated the down-regulation of GPC3[61,62,63], other investigations on hepatocellular carcinoma have shown a marked elevation of GPC3 mRNA over the level observed in corresponding normal tissues[64,65,66,67] and GPC3 exhibits a diffuse and uniform distribution within individual tumors. The expression appeared to bear no relation to the size of the hepatocellular carcinoma and was observed even in small-sized tumors[68]. Besides, up-regulation of GPC3 in hepatocellular carcinoma can also be observed at the protein level[69]. Capurro et Al.[70] observed that by staining fixed tissue sections, 72% hepatocellular carcinoma sections display GPC3 expression, whereas this protein was undetectable in hepatocytes from normal liver and benign liver diseases. Moreover, by using an ELISA, they found that whereas GPC3 is undetectable in the serum of healthy donors and patients with hepatitis, its levels were significantly increased in 53% with hepatocellular carcinoma. Similar results have also been reported independently by two other groups[71,72]. Based on these results, it has been proposed that GPC3 could be used as a serum and histochemical marker for hepatocellular carcinoma[69,71] and it was demonstrate that GPC3 forms a complex with Wnts. Glypican-1 displays high levels of expression throughout the brain and skeletal system[73], in which the major expression is observed in osteoblast, developing skeletal and smooth muscle. To note that GPC1 expression is very little in embryonic cardiomyocytes, but strong detected in neonatal and adult heart of Drosophila[74]. GPC1 has an important role in the pancreatic cancer cell invasion and metastasis, because it was showed that glypican-1 and syndecan-1 are shedded by these cancer cells[75,76,77] and GPC1 down-regulation suppresses pancreatic cancer cell growth and slightly modifies signaling of members of the TGF-beta family of growth factors[78]. GPC2 expression is restricted to the developing neuronal tissue, like

brain, spinal cord, dorsal root ganglia and cranial nerves and is not present in the corresponding adult tissue; its down-regulation seems to follow the migration and maturation of neurons[79]. GPC4 show a limited pattern of embryonic expression[36]; in developing brain glypican-4 is highly produced by neural precursor cell and its expression fall off during differentiation[80]; is present at high levels in the developing kidney and regulates hepatocyte growth factor-mediated morphogenesis in renal epithelial cells[81]. Briefly, GPC5 is found in restricted number of organs in embryo[38] and during early development glypican-5 is expressed with the onset of mesenchymal proliferation and condensation. GPC5 expression is very limited in developing central nervous system, but in adult its expression is more widespread. GPC6, like GPC5, is found in condensing mesenchyme surrounding bronchi (i.e.)[37] and strong expression is observed also in other district, like developing gastrointestinal tract or in subendothelial smooth muscle cell layers of major blood vessels[37]. Although, in general, the expression of glypicans seems to be involved in cell proliferation and migration, the ways with which they acts are more complex, for example GPC1 and GPC4 are involved in proliferation of neural precursor cell, while GPC2 seems to inhibit this process[61,63,64]. Moreover some proteins of this family can play a double role, because they can acts both stimulating proliferation and like tumour suppressor of the ectopic signals produced by either their loss- or gain-of function[82] in relation to tissue or cellular context[62] (Figure 6).



GLYPICAN-2



GLYPICAN-4/GLYPICAN-6

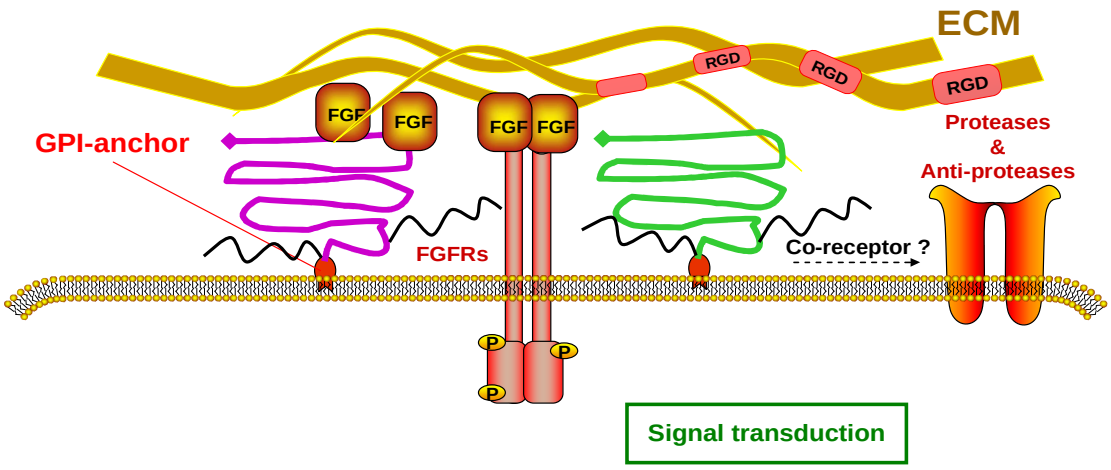


Figure 6: Schematic overview of the putative molecular functions of the GPI-linked PGs; similarly to syndecans through their HS chains they are believed to sequester a variety of growth factors and they are postulated to be involved in cell-ECM interactions

1.3.1 GLYPICAN-5

Glypican-5 was identified and characterized the first time using the strategy of screening the partial cDNA sequences available in the expressed sequence-tagged database (dbEST)[83] and was showed that this new member of glypican family had all the structural features characteristic of the other glypicans (Figure 7).

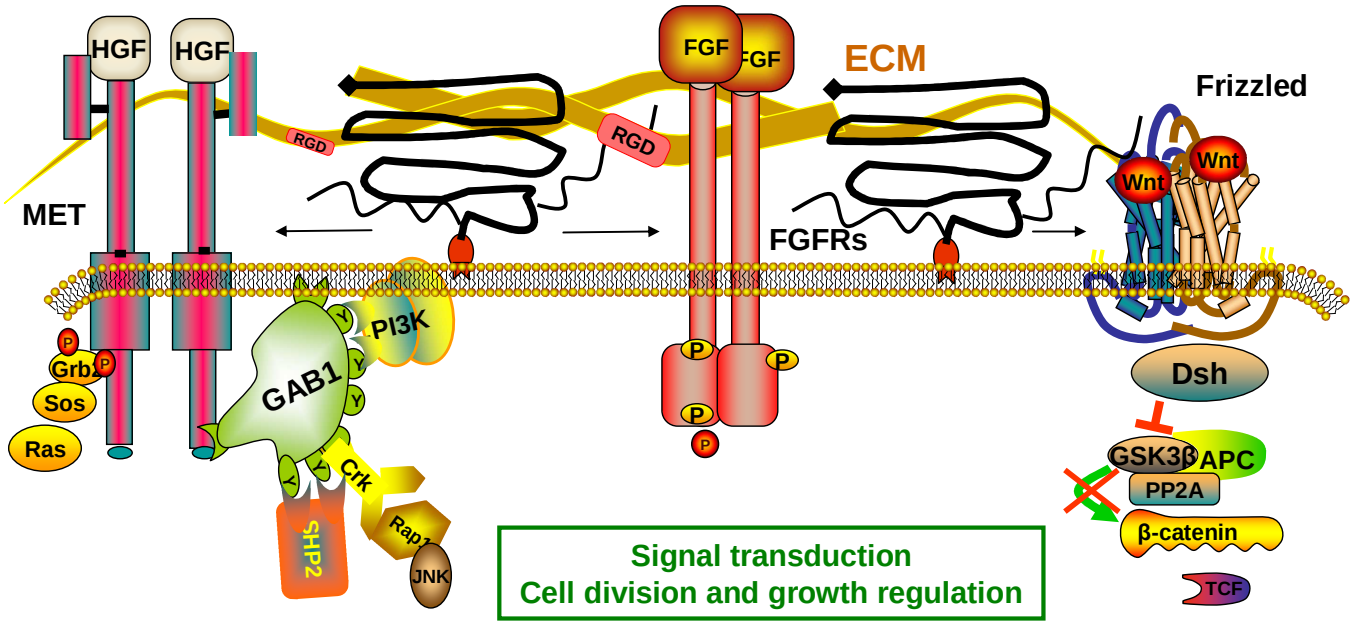


Figure 7: Schematic overview of the putative molecular functions of glypican-5.

Glypican-5 is localized on chromosome 13q32, the same chromosomal band containing glypican-6 gene, and is most closely related to glypican-3: GPC3 and GPC5 are similar in C-terminal glycanation domain where the Ser-Gly repeat sequences appear split by a cluster of acidic residues that are flanked by cysteines (to form an acidic bulge on the protein); this is in contrast of GPC1 and GPC4 that have this Ser-Gly repeat sequences in front of this bulge[46]. Clustering of the GPC5/GPC6 genes on chromosome 13q32 is strongly reminiscent of the clustering of the GPC3/GPC4 genes on chromosome Xq26 and suggests GPCs arose from a series of gene and genome duplications. Based on

similarities in sequence and gene organization, glypican-1, glypican-2, glypican-4, and glypican-6 appear to define a subfamily of glypicans, differing from the subfamily comprising so far glypican-3 and glypican-5[37] (Figure 8).

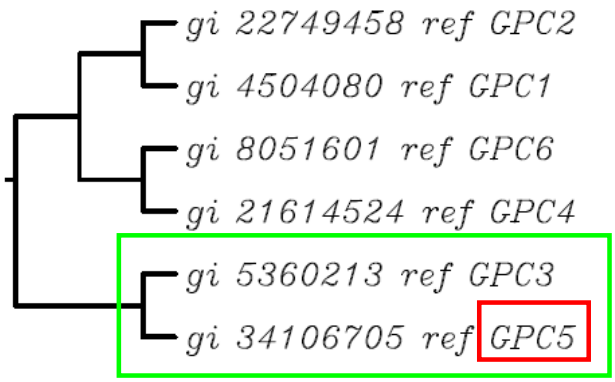


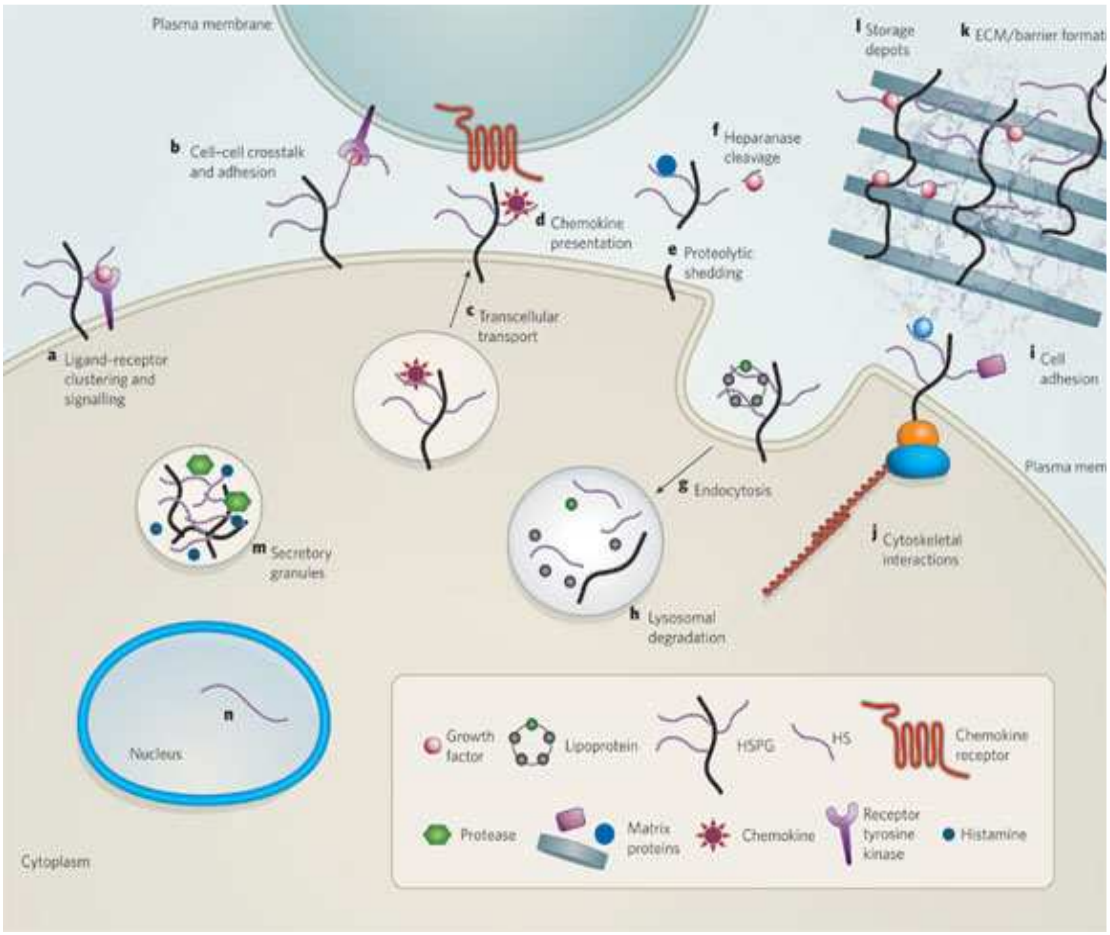
Figure 8: Glypican family phylogenetic tree. Multiple Sequence Alignment by CLUSTALW

Glypican-5 is expressed, like other glypicans, in highly tissue-specific manner during embryogenesis. In the earliest stage of mouse embryo limb, GPC5 mRNA is localized to highly proliferative cells that are in the process of generating differentiated structures and GPC5 expression becomes restricted when undifferentiated, proliferative mesenchyme of the developing limb bud starts to become differentiated skeletal and connective tissue elements. Like this, glypican-5 mRNA expression is localized in highly proliferative cells in kidney; although this observation link GPC5 expression with proliferative cells in limb and kidney embryo, glypican-5 function is unrelated with proliferation because in the cell layers of the central nervous system of embryo where are confined the most neural proliferation, GPC5 expression is missed[38]. In general, GPC5 expression marks, in the early nervous system, distinct subset of newly generated postmitotic neurons; only in later embryonic and perinatal life does glypican-5 begin to be widely expressed throughout the brain[38]. These shift in the expression of glypican-5 during embryogenesis are different, and in some sense complementary to, those showed from other glypicans: glypican-2 is expressed in developing neurons until they become postmitotic an then its expression rapidly disappears[39,63]. Glypican-1 mRNA expression seems appears later than GPC2 expression, but its expression level increase in adult brain[84]. Saunders et al.[38] hypothesize a tidy model of temporal expression: in developing neurons, first appears

GPC2 expression, then GPC5 and at least are predominantly GPC1 expression; the significance of this pattern remain still unclear. Deletions instead in the long arm of chromosome 13 (where map glypican-5 and glypican-6 gene) was associated with developmental abnormalities known as 13q- syndrome[85,86] and glypican-5 seems to act, like glypican-3, as indirect regulator of cell proliferation and a direct regulator of differentiation. Glypican-5 is also implicated as possible target for the 13q31-q32 amplification detected in lymphoma cell lines[87] where, through CGH (comparative genomic hybridization) analysis have detected gains of copy number on this chromosomal region and GPC5 is over-expressed and it may contributes to development and/or progression playing an important role in pathogenesis of lymphomas, in mantle cell lymphoma (MCL, an aggressive non-Hodgkin's lymphoma) and multiple B-cell lymphomas[88,89] and other tumors[71]. Recently, it was reported that genomic amplification of 13q31-q32 is associated also with rhabdomyosarcomas; genomic copy number and gene expression analyses of rhabdomyosarcomas indicate that GPC5 is the only gene consistently expressed and up-regulated in all cases with amplification[90].

1.4 CELL SURFACE HSPGs SIGNALLING AND INTERACTION WITH THE ECM

HSPGs are present on the surface of every eukaryotic cell, including both tumour cells and cells that are important for tumour survival. Heparan-sulphate proteoglycans on the tumour cells surface have been shown to be important in many aspects of tumour phenotype and development, including cellular transformation, tumour growth, invasion and metastasis. Specifically, certain HSPGs sequences that are expressed on the tumour cell surface seems to be pro-tumorigenic; by dynamically regulating the composition and sequence of HSPGs, cancer cell modulate their growth kinetics and metastatic potential. HSPGs on the surface of tumour cell seems to mediate their effects by regulating growth factor signalling, cell adhesion, proliferation and migration and angiogenesis[91,92] (Figure 9).



From: Bishop, J.R., Schuksz, M. & Esko, J.D. Nature 446, 1030-1037(26 April 2007)

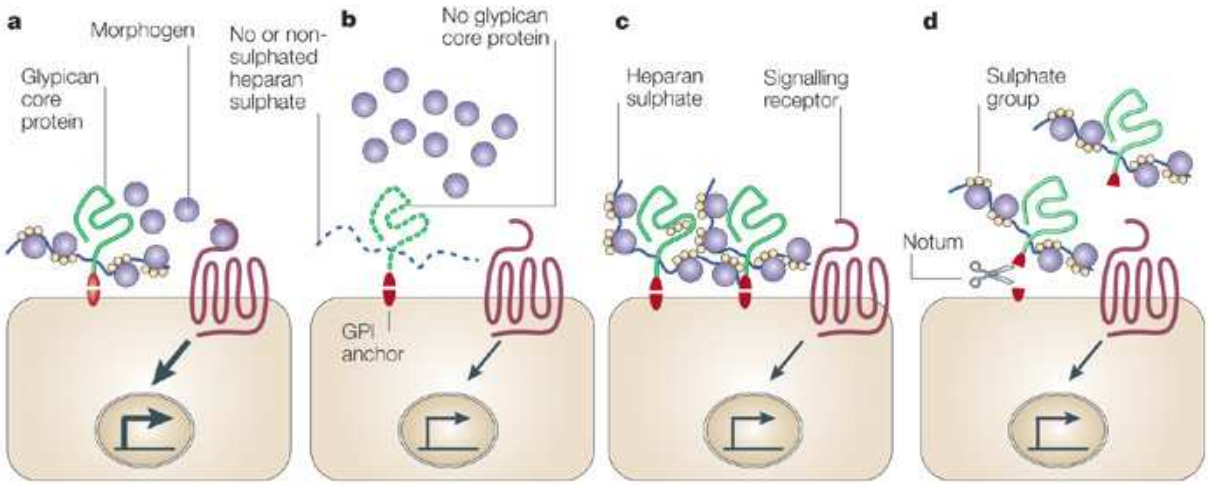
Figure 9: HSPGs function as co-receptors for growth factors and their receptor tyrosine kinases, which are present either on the same cell a) or on adjacent cells b). They transport chemokines across cells c) and present them at the cell surface d). A proteolytic processing lead to the shedding of syndecans and glypicans from the cell surface e), and heparanase cleaves the HS chains f), liberating bound ligands (such as growth factors). Cell-surface HSPGs are actively taken up by endocytosis g) and can recycle back to the surface or be degraded in lysosomes h). HSPGs also facilitate cell adhesion to the extracellular matrix i) and form bridges to the cytoskeleton j). Secreted HSPGs are involved in the formation of organized extracellular matrices that form physiological barriers k) and sequester growth factors and morphogens for later release l). Serglycin carrying highly sulphated heparin chains is packaged into secretory granules of hematopoietic cells m). Finally, some experiments suggest that HS chains exist in the nucleus n), although their function in this location is unknown.

As a consequence, alteration in the level of expression of the core protein, as well as GAG structure and/or density on HSPGs, can potentially make cancer cell highly versatile in modulating their behaviour[75]. Based on the information about the immense structural diversity of HSPGs, these molecules are able to bind and interact with a wide variety of proteins, such as growth factors, chemokines, morphogens and enzymes. Growth factors, such as fibroblast growth factors (FGF1 and FGF2), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor-β (TGFβ) and platelet-derived growth factor (PDGF)[93] have an important role in signalling mediated by HSPGs, particularly growth factor signaling seems to be delicately controlled by an auto-regulatory loop involving the syndecan and glypican expression levels on the cell surface and their role in endocytosis of receptor-ligand complexes[94,95,96]. Several classes of growth factors, including heparin-binding members of the EGF family, such as heparin-binding EGF-like growth factor (HB-EGF), HGF, Wnts, Hedgehogs and some members of the TGFβ superfamily seem to dependent upon the interaction with heparan sulfate chains of surface PGs for optimal triggering of signal transduction inside the cell[52,97,98,99]. These specific interactions allow HSPGs to "fine-tune" protein function and regulate diverse biological processes[75]. The unique structural complexity of tumour-cell HSPGs, enable them to modulate directly and/or indirectly several aspects of tumour-cell phenotype, including growth kinetics, invasiveness and metastatic potential[75,76]. In this way, a specific pattern of a cell surface HSPGs can be pro-tumorigenic or anti-tumorigenic depending on whether present in the ECM can binds the GAGs chain of HSPGs or on whether in the ECM can binds the cell surface receptors, which link membrane HSPGs as co-receptor. Recently, it was highlight that cancer cell, as a part of transformation process, alter their cell surface heparan-sulphate proteoglycans profile including differential expression of particular proteoglycan core protein sequences[100]; glypicans have also been found to modify cellular response to bone morphogenetic protein (BMP)[101] and insulin-like growth factors-2 (IGF-2) that are important in cellular division and differentiation[75]. Other important role involved HSPGs in regulating morphogen gradients, where unknown heparan-sulphate proteoglycan affect Indian hedgehog (IHH), a protein that control proliferation, hypertrophy and ossification of chondrocytes[1]. Particularly, syndecans can "cross-talk" with other cells or with

element of extracellular environment and transduct directly the information into the cell through their intracellular domain. Otherwise, glypicans cant' operate directly in signal transduction, because they lack the intracellular domain; both syndecans and glypicans can mediate the passage of the information from out to in of the cell acting vicariously as co-receptor with cell membrane receptor for signal molecules. Other ways which syndecans and glypicans can relay our contribute to signal transduction are their shedding in the extracellular environment or their internalization within the endocytotic vesicle, in which the GPI-anchored protein can be endocytosed and degraded, cleaved and released, vesiculated, or exchanged onto another cell[102]

1.5 SIGNALING MEDIATED BY GLYPICANS

The GPI anchor is one of crucial elements that could make glypicans susceptible to additional post-translational modifications. Particularly, the GPI anchor appears to provide a system of regulated release of glypicans to the extracellular environment: the lipid anchor can be removed by protease or lipase leading the shedding of them from the plasma membrane to generate either soluble or glypican form associated with low-density particles[66]. Proteolytic cleavage of the core protein can also contribute to generate distinct glypicans form. Once secreted, regulatory signal peptides bind to cell membrane receptors and activate specific intracellular cascades, thus determining cell fate[103]; the extracellular signals can act both at short and long distances by either acting on cells near the producing source or on those more distant to it. As consequence of it, short- and long-range signaling trigger distinct biological outcome according to the spatial position of targeted cells[87]. There are different enzyme that can cleave glypicans GPI anchor from cell surface, and two of them are endogenous GPI-PLD (phospholipase D) and one of recent discovery called Notum (an alpha/beta-hydrolase)[54,55,104] (Figure 10) is to underlined that mammalian Notum is active in the extracellular environment and has a cleavage site in the GPI anchor similar to that of GPI-PLC (phospholipase C)[56]; on the contrary of Notum, GPI-PLD seems to be active only in the intracellular environment[53,56,88,105].



From: Häcker, U., Nybakken, K., & Perrimon, N. Nat. Rev. Mol. Cell Biol. 6, 530-541 (July 2005)

Figure 10: a) Glypicans function as low-affinity binding proteins for secreted morphogens. Glypicans maintain secreted signalling molecules at the cell surface at levels that are sufficient to activate high-affinity signalling receptors and ensure activation of target genes. b) In the absence of glypican core protein or sufficiently sulphated glycosaminoglycan (GAG) chains, morphogen levels at the cell surface are reduced and the activity of signal transduction pathways is diminished. c) When glypicans are over-expressed, morphogen levels at the cell surface are elevated owing to the increased binding capacity of the additional GAG chains. Signalling activity might be reduced owing to a shift of the equilibrium from signalling receptor activation to GAG binding. d) Glypicans can be shed from the cell surface by Notum-mediated cleavage of the glycosyl-phosphatidylinositol (GPI) anchor. Shedding of glypicans might locally reduce morphogen activity at the cell surface and result in decreased activity of signal transduction

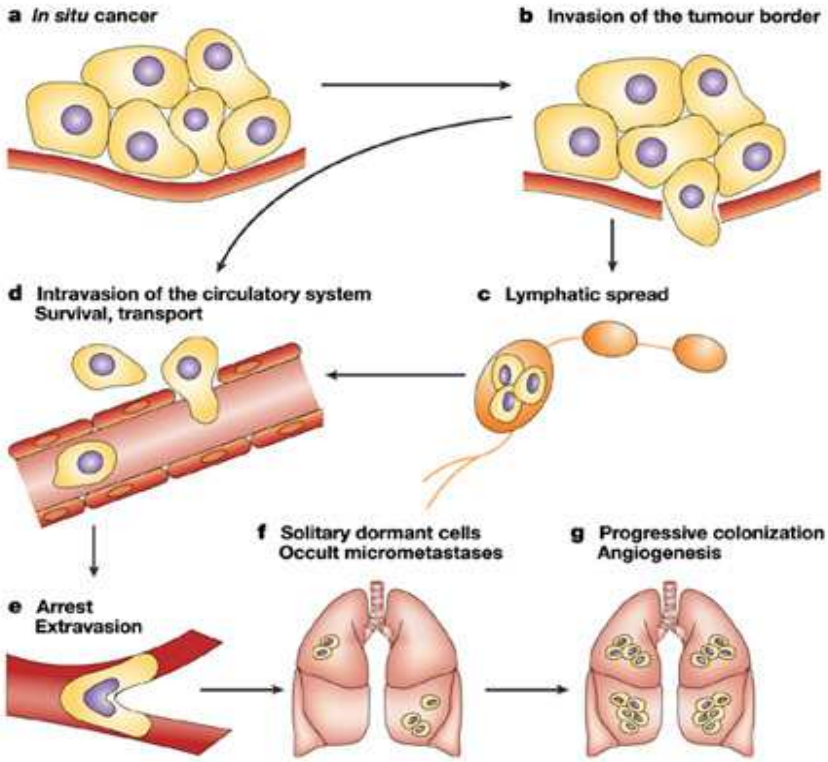
Glypicans can be act in signal transduction also from inner of the cell after their internalization: the endocytic mechanism employed can be different, as association with lipid raft, caveolar uptakes, clathrin-mediated endocytosis or macropinocytosis and most probably could be depend as the nature and distribution of proteoglycans expressed on the cell surface. Glypicans are preferentially associated with lipid-raft, detergent-resistant microdomains enriched in relatively satured phospholipids, glycosphingolipids and cholesterol or in caveolae, which are specialized forms of lipid raft[106,107]. It is now becoming clear that lipid micro-environments on the cell surface also take part in the process of signal transduction because it is initiated by complex protein–protein interactions between ligands, receptors and kinases, for example. Lipid rafts containing a

given set of proteins that can change their size and composition in response to intra- or extracellular stimuli. This favours specific protein–protein interactions, resulting in the activation of signalling cascades[108]. How glypicans internalized can act in subsequent signaling pathways are still unknown.

1.6 TUMOUR SPREAD AND METASTASIS FORMATION

Metastasis, the spread of cancer from the primary tumor to circulation and other parts of the body remains a prominent problem in the search for a cure for cancer. The most deadly aspect of cancer is its ability to spread, or metastasize. Cancer cells initially group together to form a primary tumor. Once the tumor is formed, cells may begin to break off from this tumor and travel to other parts of the body. To metastasize, a cancer cell must break away from its tumor, invade either the circulatory or lymph system, which will carry it to a new location, and establish itself in the new site. Metastasis most commonly occurs by way of the bloodstream or the lymphatic system. Once in the bloodstream, the cancer cells now have access to every portion of the body. The lymphatic system has its own channels throughout the body like the circulatory system, through which a malignant cell can travel. These cancer cells that travel through the body are capable of establishing new tumors in locations remote from the site of the original disease[109](Figure 11). Metastasis causes 90% of deaths from solid tumours and displays a remarkably diverse set of clinical manifestations. The risk of metastatic recurrence can sometimes be predicted from certain features of the primary tumour, as in the case of breast cancer, for which tumour size, histological grade, and gene-expression pattern are indicative of the risk of relapse. In other instances, metastatic disease presents itself with an unknown primary tumour source, rendering such correlations impossible to make. Moreover, disseminated tumour cells could theoretically also return to their original site (self-seeding), thereby accounting for the progressive accumulation of aggressive cells in primary tumours and local recurrences. Work over the past three decades has identified many genes for which gain- or loss-of function confers autonomous proliferative activity, resistance to cell death cues, angiogenesis, altered cell adhesion and motility. In other

words, genetic alterations that mediate the initiation and local progression of tumours, and that collectively confer the prerequisites for metastasis, have been identified[109].



From: Steeg, P.S. Nature Reviews Cancer 3, 55-63 (January 2003)

Figure 11: A schematic of the metastatic process, beginning with a) an *in situ* cancer surrounded by an intact basement membrane. b) Invasion requires reversible changes in cell–cell and cell–extracellular-matrix adherence, destruction of proteins in the matrix and stroma, and motility. Metastasizing cells can c)| enter via the lymphatics or d) directly enter the circulation. e) Survival and arrest of tumour cells, and extravasation of the circulatory system follows. f) Metastatic colonization of the distant site progresses through single cells, which might remain dormant for years, to occult micrometastases and g) progressively growing, angiogenic metastases.

However, we are only beginning to learn about the genetic determinants of metastasis proper; that is, those that mediate tumour cell invasion, intravasation, survival in circulation, scattering to distant tissues, extravasation into parenchyma, and colonization of vital organs[95]. Glypicans can have a crucial role in this context for their multiple roles

that can play in each steps of the metastatic process for their biological characteristic showed previously.

Research is now focused on understanding in what ways cancer cells become potentially metastatic and aim to identify new cancer-associated gene-expression signatures as new tools to classify tumour subtypes, discern disease aetiology, predict risk of relapse, identify genes that mediate disease progression, and select optimal therapeutic options.

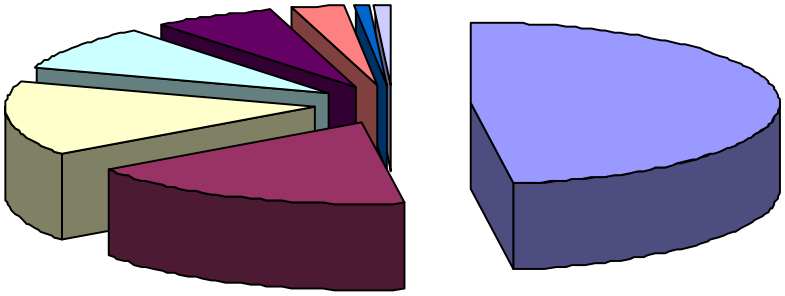
1.7 MUSCULOSKELETAL SARCOMAS

Bone and soft tissue malignant tumors are also called musculoskeletal sarcoma, which means a cancer of mesenchymal tissues, such as the bone, soft tissues, and connective tissue. Bone cancer is a relatively rare disease in which cancer cells grow in the bone tissue. Cancer may form in the bone or spread to the bone from another site in the body. When cancer starts in bone tissue, it is called primary bone cancer. When cancer cells travel to the bone from elsewhere, it is called secondary or metastatic cancer to the bone
Primary tumors of bone can be divided into benign tumors and cancers. Common benign bone tumors may be neoplastic, developmental, traumatic, infectious, or inflammatory in etiology. Examples of benign bone tumors include osteoma, osteochondroma, aneurysmal bone cyst, and fibrous dysplasia. Malignant primary bone tumors include osteosarcoma, chondrosarcoma, Ewing's sarcoma, and other sarcoma types. Multiple myeloma is a hematologic cancer which also frequently presents as one or more bone tumors. The most common types of bone cancer include:

- Osteosarcoma — a cancerous tumor of the bone, usually of the arms, legs, or pelvis; osteosarcoma is the most common primary cancer.
- Chondrosarcoma — cancer of the cartilage; chondrosarcoma is the second most common primary cancer.
- Ewing's sarcoma — tumors that usually develop in the cavity of the leg and arm bones

- Fibrosarcoma and malignant fibrous histiocytoma — cancers that develop in soft tissues (e.g., tendons, ligaments, fat, muscle) and move to the bones of the legs, arms and jaw
- Giant cell tumor — a primary bone tumor that is malignant (cancerous) only about 10% of the time; most common in the arm or leg bones
- Chordoma — primary bone tumor that usually occurs in the skull or spine

Bone sarcoma	%
Osteosarcoma	47.75
Ewing's sarcoma	17.57
Chondrosarcoma	15
Non-Hodgkin's lymphoma	9.01
Plasma cell myeloma	6.31
Other sarcomas	2.70
Chordoma	0.90
Langerhan's cell histiocytosis	0.90



Modified from: Cancer Path. Reg. '03-'04 And Time Trend Analysis. Department of Pathology, NCI 2007

Table 5: Percentage of incidence of bone sarcomas in U.S.A.

Osteosarcoma and Ewing's sarcoma are two of the most common types of bone cancer and generally occur in children and young adults. Chondrosarcoma is cancer of the cartilage and is more common in adults. Chordoma is a type of bone cancer that typically starts in the lower spinal cord. Rare, soft-tissue sarcomas that begin in the bone include malignant fibrous histiocytoma (MFH) and fibrosarcoma. MFH makes up less than 1% of primary bone tumors. It is usually found in adults. The arms and legs, especially around the knee joint, are the most common sites for MFH to appear. Fibrosarcoma is also more common among adults, particularly during middle age, and most often begins in the thighbone. In 2008, an estimated 2,380 adults (1,270 men and 1,110 women) in the United States will be diagnosed with bone cancer. It is estimated that 1,470 deaths (820 men and 650 women) from this disease will occur this year. Primary bone cancer accounts for less than 0.2% of all cancers. Osteosarcoma makes up 47% of all primary bone cancers, followed by Ewing's family of tumors (17.5%), chondrosarcoma (15%),

chordoma and MFH/fibrosarcoma less than 5%[110]. Soft tissue sarcoma (STS) is cancer that develops in the tissues that support and connect the body[111]. Large majority of soft tissue tumours are benign, with a very high cure rate after surgery. Malignant mesenchymal neoplasms amount to less than 1% of the overall human malignant tumours, but are very hard-working to diagnose them and study their therapeutic protocol since there are more than 50 histological subtypes of STS. Benign mesenchymal tumours outnumber sarcomas by at least 100 to one[112,113]. Unlike the more common carcinomas, which are of epithelial origin, soft tissue sarcomas are of putative mesenchymal derivation and can involve connective tissue structures as well as viscera and integument anywhere in the human body, but about 40% occur in the arms or legs, 36% occur in the trunk or abdomen, and 11% occur in the head or neck[112] whereas the percentage rest are located in the trunk wall, retroperitoneum, abdomen or other location[114] (Figure 12).

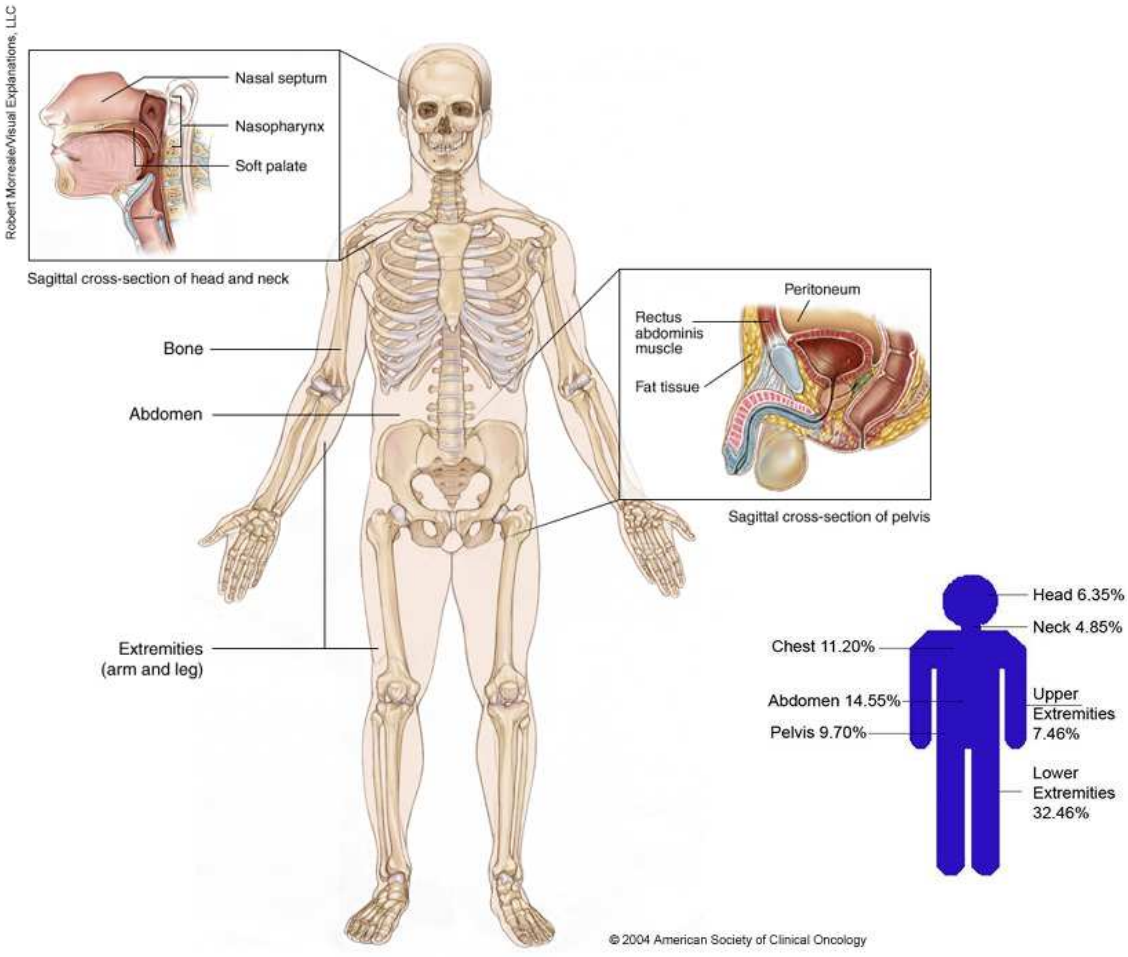


Figure 12: Soft Tissue Sarcoma anatomical site and % of distribution

Cancer begins when cells grow out of control, and instead of developing into normal cells, they form a tumor mass (lump). When sarcoma is small, it can go unnoticed or is ignored, since it does not usually cause problems at this stage. As sarcoma grows, it can interfere with the body's normal activities; it can also spread to other places in the body. STS can appear in any part of the body. The specific types of sarcoma are frequently named according to the normal tissue cells they most closely resemble (Table 6), rather than referring to an anatomical site (part of the body) as many other cancer types are named.

Table 6: Types of Soft Tissue Sarcomas

Name of STS	**Tissue type**
Angiosarcoma	Blood or lymph vessels
Ewing's sarcoma/PPNET (peripheral primitive neuroectodermal tumor)	Mesenchymal stem cells
Fibrosarcoma	Fibrous tissue
Gastrointestinal stromal tumor (GIST)	Interstitial cells of the digestive tract
Kaposi's sarcoma	Blood vessels
Leiomyosarcoma	Smooth muscle
Liposarcoma	Fat tissue
Myxofibrosarcoma (myxoid malignant fibrous histiocytoma [MFH])	Connective tissue
Neurofibrosarcoma or malignant peripheral nerve sheath tumor	Peripheral nerve
Pleomorphic sarcoma, not otherwise specified (NOS)	Unknown
Rhabdomyosarcoma	Skeletal muscle
Synovial sarcoma	Unknown

Experts have identified many types and subtypes of sarcoma. Pathologists are now trying to find new ways to quickly determine a tumor's subtype, as this helps determine treatment. The definition of staging, hystotypes and the distribution of these varies over time and between researchers: the age-related incidence vary; embryonal rhabdomyosarcoma occurs almost exclusively in children, synovial sarcoma mostly in young adults, whereas pleomorphic high grade sarcoma, liposarcoma and leiomiosarcoma dominate in the elderly[98,115]. Most STS have no clearly defined etiology;

in rare case, genetics, irradiation, viral infections and immune deficiency have been found associated with the development of usually malignant soft tissue tumours; however, the large majority of soft tissue sarcomas seem to arise *de novo*, without an apparent triggering event[112]. Genetic mutations in pluripotent mesenchymal stem cells are believed to give rise to malignant clones, which lead to the formation of these disease types[114]. The determination of staging play a crucial role in the diagnosis: the American Joint Committee on Cancer (AJCC) have developed a Staging System in parallel with other cancer society; there are other factor that contributes in the diagnosis determination, among which the prognostic factors: site of primary tumor, the margin status of the resected tumor, size of STSs, if the tumor is *de novo* or recurrent lesion and finally but not back-burner the possible presence of molecular staging/prognosis markers. In a century of analysis of molecular biology of cancer, in that we can find a key to understand one of the elements of the tumour cascade in which we can use to fight back. Examination of a tumor's abnormal genetic code may help determine its characteristics and predict which treatments will be most effective. For at least one type of sarcoma, gastrointestinal stromal tumor (GIST), major advances have been made in targeted therapy (drug treatments targeted to specific genetic abnormalities in the sarcoma cell). Sarcoma is rare, accounting for about 1% of all cancers. In 2008, approximately 10,390 people (5,720 males and 4,670 females) will be diagnosed with soft tissue sarcoma in the United States. An estimated 3,680 adults and children (1,880 males and 1,800 females) are expected to die of the disease this year. The overall five-year relative survival rate (percentage of patients who survive at least five years after the cancer is detected, excluding those who die from other diseases), combining all stages and types of sarcoma, is approximately 66% and the key determinant of survival is control of both local recurrence and as well as distant dissemination[114]. If the sarcoma is diagnosed at an early stage in an arm or leg, the five-year survival rate is 81% to 90%. If the sarcoma has spread or is located in another part of the body, then the five-year relative survival rate is much lower. Cancer survival statistics should be interpreted with caution. These estimates are based on data from thousands of cases of this type of cancer, but the actual risk for a particular individual may differ. It is not possible to tell a person how long he or she will live with sarcoma. Because the survival statistics are measured in five-year (or

sometimes one-year) intervals, they may not represent advances made in the treatment or diagnosis of this cancer[97].

1.7.1 MUSCULOSKELETAL SARCOMAS AND GLYPICANS

Generally tumorigenesis is associated with changes in the PG synthesis. Heparan sulfate PGs are involved in several aspects of cancer biology including tumor progression, angiogenesis, and metastasis. Many functions of glypicans depend on their ability to bind and modulate the activity of components of the extracellular matrix (ECM). The ability of them to interact with other molecules, such as growth factors, is largely determined by the fine structure of the glycosaminoglycan chains. Glypicans can have both tumor promoting and tumor suppressing activities depending on the protein core, the GAG attached, molecules they associate with, localization, the tumor subtype, stages, and degree of tumor differentiation. Glypicans may promote local cancer cell growth in some cancer tissues, but inhibit tissue invasion and metastasis in others. HSPG degrading enzymes like heparanase, heparitinase, and other enzymes such as MMP are also important in tumor metastasis[116]. There are very few information that can cross-link Soft Tissue Sarcomas and glypicans: the scientific literature reports only careful study about glypican-3 and one case regarding glypican-5 of our interest. Several studies have shown that glypican 3 (GPC3) could be a useful diagnostic marker for hepatocellular carcinoma (HCC) and for differentiating HCC from nonneoplastic and preneoplastic liver disease. Furthermore, several other tumors revealed consistent expression of GPC3, including squamous cell carcinoma of the lung (27/50 [54%]), testicular nonseminomatous germ cell tumors (32/62 [52%]), and liposarcoma (15/29 [52%])[117]. GPC3 is also indentified as a novel important marker transcript that have not been previously associated and that can clearly discriminate between pediatric rhabdomyosarcomas (RMS) and Ewing's sarcomas (EWS), because the diagnostic classification of these cancers is frequently complicated by the highly similar appearance in routine histology, and additional molecular markers could significantly improve tumor classification[118]. Furthermore, in rhabdomyosarcomas it was show that there is a region of amplification at 13q31-32, a genomic region that

containing two genes: C13orf25 and GPC5[90,119,120]; it was also demonstrate that constitutive overexpression and knockdown of GPC5 expression in this type of pediatric sarcoma cell lines increased and decreased cell proliferation, respectively[90], and it was showed that glypican-5 increases proliferation through potentiating the action of the FGF2, HGF and Wnt1A[121]; GPC5 enhanced the signaling cascade of FGF2, that leads to mesodermal cell proliferation without induction of myogenic differentiation, and HGF and altered the cellular distribution of the first; for this reason, it is possible that GPC5 may influence growth factor signaling by retaining GFs either at the cell surface or by facilitating internalization[90,121]. This genomic amplification is an important element in cancer, because overexpression of gene through this model of action and other mechanisms can critically affect the behavior of tumour cells. This characteristic chromosomal imbalance, genomic amplification at 13q31-32, is frequently seen across broad range of tumor types[122], including other sarcomas with poor prognosis like liposarcomas[123].

1.8 ORAL SQUAMOUS CELL CARCINOMA

Oral cancer holds the eight positions in the cancer incidence ranking worldwide and oral squamous cell carcinoma (OSCC) representing in US 5% of all cancer for men and 2% for women[124] and affects about 30,000 Americans each year. Over 95% of the OSSC are attributable to smoke, drink alcohol, or both. OSSC represent the most common cause of death for patients with oral cancer and have an important impact on physical appearance and on the ability to eat and speech. Loco-regional relapses determine a significant decrease of the quality of life in patients with important impairment on physical, functional and social domains due to the treatment of the primitive tumour[111] (Figure 13).

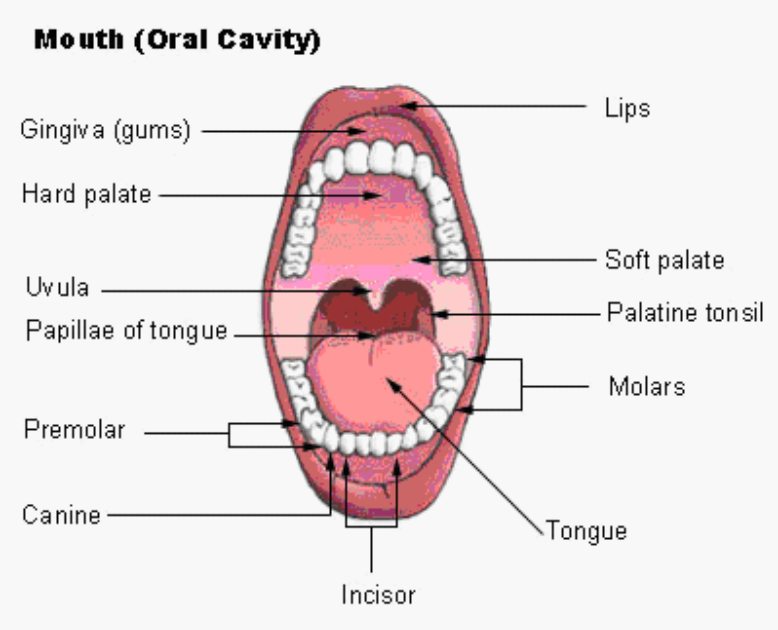


Figure 13: Oral cavity

The chief risk factors for oral squamous cell carcinoma are smoking (especially > 2 packs/day) and alcohol use. The combination of heavy smoking and alcohol abuse is estimated to raise the risk 100-fold in women and 38-fold in men. Squamous cell carcinoma of the tongue may also result from any chronic irritation, such as dental caries, overuse of mouthwash, chewing tobacco, or the use of betel quid. Oral human papillomavirus (HPV), typically acquired via oral-genital contact, may have a role in etiology[125]. About 26% of the cases are detected on the tongue. An additional 23% of cases arise from the oral portion of the pharynx. The oropharynx includes the tonsillar pillars, the soft palate, and the posterior wall of the pharynx. The lip accounts for an additional 20% of SCC cases. Most of these arise on the red portion of the lip (the vermilion). Another 17% of SCC cases arise in the mucosa on the floor of the mouth. The gingiva (9%), buccal mucosa (3%), and hard palate (2%) collectively give rise to 14%. It is important to know that the visible part of the tongue, the dorsum, is not a site where SCC develops; rather, it develops on the side (lateral surfaces) and underside (ventral surface), two areas not visible to cursory intraoral examination (Table 7).

Table 7: Incidence of SCC by location

General Location	Incidence	Specific Location
Tongue	26%	Lateral surface Ventral surface
Oral Pharynx	23%	Soft palate Tonsillar pillars
Lip	20%	Vermilion surface
Floor of Mouth	17%	Floor of mouth
Gingiva	9%	Gingiva
Buccal Mucosa	3%	Buccal mucosa
Hard Palate	2%	Hard palate

It is the lateral tongue, the ventral tongue, the soft palate, the tonsillar pillars, and the floor of the mouth that account for two-thirds of the cases of intraoral SCC. These sites are designated as "high risk areas"[126,127]. Early, curable lesions are rarely symptomatic; thus, preventing fatal disease requires early detection by screening. Treatment is with surgery, radiation, or both. The overall 5-yr survival rate (all sites and stages combined) is >50%[125]. Unfortunately, currently implemented methods to predict disease reoccurrence such as staging and grading do not show a sufficiently accurate stratification of the patients, and molecular markers to predict disease progression are limited in number and efficiency; knowing in advance which patients have the higher risk of disease reoccurrence, despite a similar staging, would be important to focus resource only in a limited high-risk subgroup of patients.

1.8.1 PROTEOGLYCANS IN OSCC

In the development of oral squamous cell carcinoma probably are involved several proteoglycans, but only for syndecan-1 there are data in the literature. In B lymphocytes and stratified squamous epithelia, syndecan-1 is proposed to function as a cell-cell adhesion molecule[128,129,130]. Furthermore, it plays an important role in the regulation of cell growth and differentiation during the developmental process[20,21]. During wound healing, syndecan-1 expression is enhanced in the proliferating epidermal cells, but it is also induced in the endothelial cells of the developing capillaries in granulation tissue[131]. In mature tissues, the expression of syndecan-1 is limited to the epithelial cells, with the exception of B cells and Leydig cells and it was previously shown that syndecan-1 expression is reduced in epidermal keratinocytes during experimental skin carcinogenesis in mice[131]. In squamous cell carcinomas in people, a marked down-regulation of syndecan-1 expression is detected in SCCs of the head and neck as well as in the uterine cervix, when compared to the expression of syndecan-1 in corresponding normal epithelium. In fact, syndecan-1 has been proposed as a prognostic marker in SCCs of the head and neck[132,133]. There are no information about relationship between glypicans and OSCC, but is necessary a deeply analysis of proteoglycan pattern of expression on tumour cells to obtain a more clearly picture if there are other PGs involved apart from syndecan-1.

2. MATERIALS AND METHODS

2.1 SURGICAL SPECIMENS

Specimens were obtained surgically from 22 patients (8 women and 14 men; ages ranged from 32 to 88 years) with OSCC by the equip of Enrico Sesenna (Maxillo-Facial Surgery Division, Head and Neck Department, University and Hospital of Parma, Parma, Italy) between 2007 and 2008 (Table 8). The normal oral epithelia (5 cases) as control were also evaluated. All specimens were aliquotated in 3 tissue cube of 1.5cm of thick and stored in RNAlater Solution (Ambion) for at last 1 day and then 2 aliquots were frozen at -80°C and one analyzed to extract total RNA to processes for subsequent Real-time quantitative PCR studies. Tumoural biopsy were histologically diagnosed following TNM classification criteria, subdivided in four classes (T1T2, T3T4, age minor or major of 40 years correlate to TNM classification) and then compared with normal mucosal epithelium.

Table 8: Surgical specimen analyzed

COD BIOPSY	TNM[1]	AGE
1	T3T4N+	41
2	T3T4N-	32
3	T3T4N+	78
4	T1T2N-	34
5	T3T4N+	61
6	T1N-	38
7	T1T2N-	43
8	T3T4N+	43
9	T1T2N+	50
10	T2T3N+	64
11	T3T4N+	79
12	T1T2N-	61
13	T3T4	57
14	T2N-	74
15	T1T2N-	63
16	T1T2N-	88
17	T3T4N+	72
18	T3T4N+	57
19	T2N-	nn[2]
20	nd[3]	70
21	nd	nn
22	nd	nn

[1]TNM classification provides a system for staging the occurrence of cancer., T refers to the primary tumour and is categorized as being 1-4 and a-d depending upon site, size and spread, N refers to the lymph nodes and they are categorized as being X, 0, 1, 2, or 3 depending upon if any, some or all are involved metastasis and +/- indicates if there are spread to lymph nodes or not without an accurate definition of how much of these are involved and M to the presence or absence of distant metastases. [2] nn: not note; [3] nd: not determined, in analysis.

2.2 CELLS

All sarcoma cells (Table 9), both established cell lines and patient-derived, the last ones obtained from surgical specimens surgically removed from patients with sarcomas by equips of CRO Institute (Centre of Oncologic Reference, Aviano (PD), Italy), were cultured in DMEM (Dulbecco's modified Eagle's medium) with low Glucose (1.0 g/L), 25 mM HEPES Buffer supplemented with Pen/Strep, L-Glutamine and 10% (v/v) FBS (fetal bovine serum).

Table 9: Sarcomas cells cultured; in blue established sarcoma cell lines; in red sarcomas cells patient-derived not yet characterized

CELLS	HYSTOTYPE
NTI-AS-1	ANGIOSARCOMA
NTI-CS-1	CHONDROSARCOMA
Hs913T	FIBROSARCOMA(metast. to lung)
NTI-FS-1	FIBROSARCOMA
NTI-FS-2	FIBROSARCOMA
HT1080	FIBROSARCOMA (connective tissue)
NTI-LMS-8	LEIOMYIOSARCOMA
NTI-LMS-1	LEIOMYOSARCOMA
NTI-LMS-2	LEIOMYOSARCOMA
NTI-LMS-3	LEIOMYOSARCOMA
NTI-LMS-5	LEIOMYOSARCOMA
NTI-LMS-6	LEIOMYOSARCOMA
NTI-LMS-7	LEIOMYOSARCOMA
NTI-LMS-9	LEIOMYOSARCOMA
NTI-LMS-10	LEIOMYOSARCOMA
NTI-LMS-11	LEIOMYOSARCOMA
NTI-LMS-12	LEIOMYOSARCOMA
SK-UT-1	LEIOMYOSARCOMA (uterus)
SK-LMS-1	LEIOMYOSARCOMA (vulva)
SW872	LIPOSARCOMA
NTI-LS-1	LIPOSARCOMA
NTI-LS-2	LIPOSARCOMA
NTI-LS-3	LIPOSARCOMA
NTI-LS-4	LIPOSARCOMA
NTI-LS-5	LIPOSARCOMA
NTI-LS-6	LIPOSARCOMA
NTI-LS-7	LIPOSARCOMA
NTI-MFH-1	MALIGNANT FIBROHISTIOCYTOMA
NTI-MFH-2	MALIGNANT FIBROHISTIOCYTOMA
NTI-MFH-3	MALIGNANT FIBROHISTIOCYTOMA
NTI-MFH-4	MALIGNANT FIBROHISTIOCYTOMA
NTI-MFH-5	MALIGNANT FIBROHISTIOCYTOMA
NTI-MFH-7	MALIGNANT FIBROHISTIOCYTOMA
NTI-MS-1	MULLERIAN SARCOMA
NTI-OS-1	OSTEOSARCOMA
SAOS2	OSTEOSARCOMA (bone)
143B	OSTEOSARCOMA (bone)

CELL LINE	HYSTOTYPE
MG63	OSTEOSARCOMA (bone)
GCT	PLEOMORPH MALIGNANT HISTIOCYTOMA
NTI-PNS-1	PNS SARCOMA
NTI-RS-1	RHABDOMYOSARCOMA
NTI-RS-2	RHABDOMYOSARCOMA
RD	RHABDOMYOSARCOMA (muscle)
A204	RHABDOMYOSARCOMA (muscle)
SJRH30	RHABDOMYOSARCOMA (muscle)
NTI-STRO-2	STROMAL SARCOMA
SW982	SYNOVIAL SARCOMA (synovium)
MES-SA	UTERINE SARCOMA (uterus)

Table 9: continue

2.3 DNA EXTRACTION

Clones genomic DNA was prepared using InstaGene^{TM}Matrix 6% (Bio-Rad Laboratories) according to the manufacturer's instructions. Qualitative PCR were optimized to test the DNA integrity and functionality amplifying the housekeeping gene RLP41, and amplifying Ampicillin resistance gene (β-lactamase) used for the selection in *E. coli* introduced into clones by transfections of pDisplay plasmids vectors using the respective primers shown in Table 10.

2.4 RNA EXTRACTION AND RT-PCR

Total RNA from each sarcoma cells was prepared by using Trizol® (Invitrogen) according to the manufacturer's instructions and RNA quality was checked by Biophotometer (Eppendorf) and 1% agarose RNase free TAE agarose gel electrophoresis. Total RNA from each biopsy was prepared by using Trizol® according to the manufacturer's instructions in combination with Qiagen RNAeasy Mini Kit (Qiagen) and RNA quality was checked by Thermo Scientific NanoDrop^{TM} Spectrophotometers, 1% agarose RNase free TAE agarose gel electrophoresis and Micro-electrophoresis run (Bioanalizer, Agilent, US). Total RNA (1µg) was reverse-transcribed with the

QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Qualitative RT-PCR was performed at the beginning to check the presence of glypicans and syndecans (GPC1, GPC2, GPC3, GPC4, GPC5, GPC6, SDC1, SDC2, SDC3, and SDC4) mRNA and to test the integrity and functionality of these cDNA, and it was amplified, as internal control, RLP41 housekeeping gene using the primers listed follows (Table 10).

Table 10: Table of primers used

Gene	Forward	Reverse
RLP41	GGAGGCCACAGGAGCAGAAA	TGTCACAGGTCCAGGGCAGA
GPC1	TGCTGCCTGATGACTACCTG	GAGCACAGTAGACCAGCTTCA
GPC2	ATCTGGTGAGGGGTTGGATGA	GAGAGCCATCGGTAGATGAGG
GPC3	CCTGATTCAGCCTTGGACAT	TCCCTGGCAGTAAGAGCACT
GPC4	TGGGAACGACGTGGACTTCTTTGA	AGAAGGGACCCTGGTTTGCTTGTA
GPC5	TATCCGGTCGTTGGAAGAAC	GGTGGTCTTCATTCCATGCT
GPC6	CAACATTGAGTCGGTCATGG	ATTGTAGGGCCTGAAACGTG
SDC1	GCTCTGGGGATGACTCTGAC	GTATTCTCCCCCGAGGTTTC
SDC2	CCAGCCGAAGAGGATACAAATG	GCGTTCTCCAAGGTCATAGCTTCC
SDC3	GAGCCTGACATCCCTGAGAG	CCCACAGCTACCACCTCATT
SDC4	GTCTGGCTCTGGAGATCTGG	TGGGGGCTTTCTTGTAGATG
Ampicillin	GTGTCGCCCTTATTCCCTTT	GGCACCTATCTCAGCGATCT

To control no DNA contamination was performed a PCR with primers for RLP41 on reverse-transcribed without RNA as substrate. To quantify the exactly amount of each gene expressed in all the sample tested, it was performed a Real-time quantitative PCR using TaqMan low-density arrays (TLDA), as specified follow.

Total RNA from each sarcoma cells and from each biopsy was prepared as described in the previous paragraph. Total RNA (1µg) was reverse-transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. cDNA from RNA extracted from cell lines was quantified by spectrophotometer; cDNA from RNA obtained from surgical specimen was quantified by Biophotometer (Eppendorf). The integrity and functionality of these cDNA, was tested in qualitative RT-PCR amplifying

RLP41 housekeeping gene using the primers listed (Table 10). To control no DNA contamination was performed a PCR with primers for RLP41 on reverse-transcribed without RNA as substrate. Each TaqMan Low Density Array was designed for quantification of human genes and exploit TaqMan® technology, based on the fluorogenic 5' nuclease assay. The assays were chosen among the TaqMan Gene Expression Assay library (Table 11) and the cards were runned on ABI 7900 HT Fast Real-Time PCR System.

Table 11: TaqMan Gene Expression Assay used

Gene symbol	Protein	Assay ID
GPC1	glypican-1	Hs00157805_m1
GPC2	glypican -2	Hs00415099_m1
GPC3	glypican-3	Hs00170471_m1
GPC4	glypican-4	Hs00155059_m1
GPC5	glypican-5	Hs00270114_m1
GPC6	glypican-6	Hs00170677_m1
SDC1	syndecan-1	Hs00896423_m1
SDC2	syndecan-2	Hs00299807_m1
SDC3	syndecan-3	Hs00206320_m1
SDC4	syndecan-4	Hs00161617_m1
CSPG4	NG2	Hs00426981_m1

Gene expression was normalized to endogenous housekeeping gene 18S rRNA and it was chosen cDNA of human mesenchymal stem cells (hMSC) as sample calibrator, because all sarcomas analyzed came from mesenchymal cells, whereas cDNA of pool of RNAs extract from 5 normal oral epithelia surgical specimens was chosen as sample calibrator for the analysis of biopsy. An equal amount of input cDNA (100 ng) was used per reaction and loaded in one of the eight sample-loading port of the card. Results were analyzed using ABI PRISM 7900HT Sequence Detection System (ABI), and changes in gene expression levels were calculated using the "relative quantification method" based on the expression levels of a target gene versus a reference gene in each sample in

comparison to a sample calibrator (Figure 14) with the collaboration of Nelson Marmiroli staff, in particular with the supervision of Mariolina Gulli (Department of Environmental Science, University of Parma, Italy) .

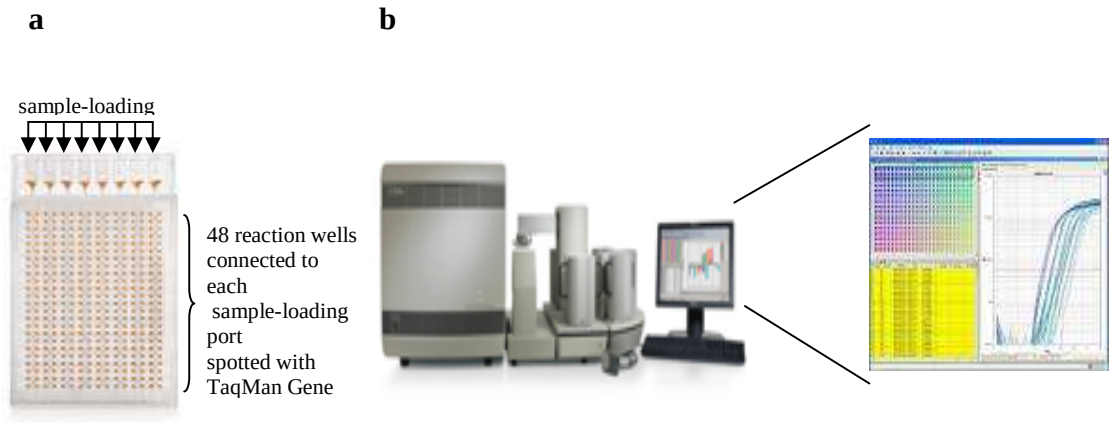


Figure 14: a) TaqMan Low Density Array; b) ABI 7900 HT Fast Real-Time PCR System.

2.5 VECTORS AND CELL TRANSFECTION

143B cells was transfected with the two types of expression vectors: pDisplay vector contained cDNA of glypican-5 Hemagglutinin A (HA)-tagged and pDisplay empty vector. The expression vector pDisplay containing ORF cDNA of GPC5 was gently provided by Guido David (University of Leuven-CME) and containing the box for G418 resistance (Geneticin/Neomycin) (Figure 15). Both 143B stably transfected cells were cultured in DMEM (as before described) and G418 [800µg/ml].

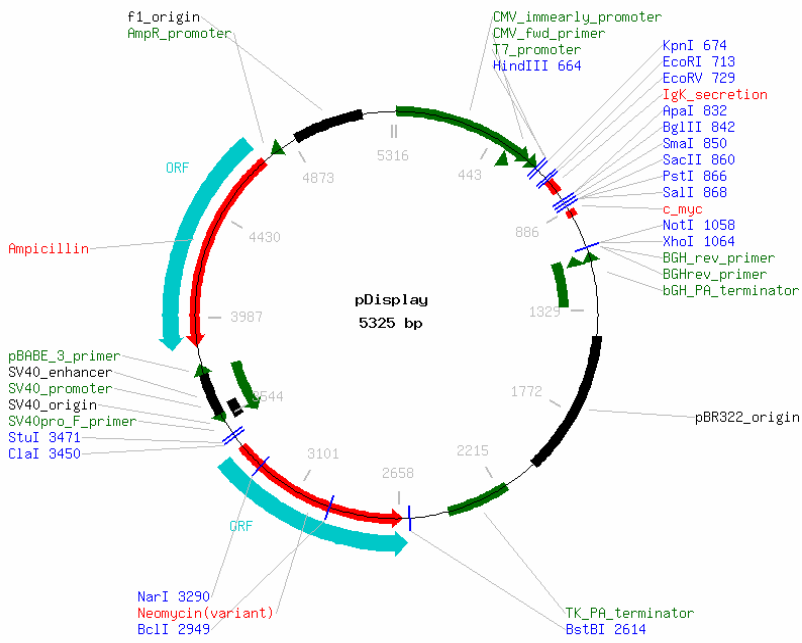


Figure 15: pDisplay vector

The transfection was done using Metafectene™ Pro Reagent (Biontex, Germany) in condition predetermined to obtain maximum efficiency of transfection (Solution A: 0.8 µg of vector DNA in 50µl medium free of serum and antibiotics, Solution B: 8 µl of Metafectene™ Pro transfection reagent in 50µl medium free of serum and antibiotics and both solutions applied on 60-70% cell confluence) and was performed an antibiotic monoclonal clones selection by G418 (to determinate the right concentration of antibiotic it was performed a dose-response curve at different concentration of G418 from 100ug/ml to 1mg/ml; he lower concentration which kill all cells in two weeks was [500µg/ml]; the clones were screened by PCR on DNA to amplify ampicillin gene and positive clones to ampicillin genes were screened further by RT-PCR for presence of GPC5 mRNA and ampicillin mRNA on positive clone in selection for respectively over-expression of GPC5 and empty vector (Figure 16). Positive clones were expanded and it was chosen the clone with the major level of glypican expression revealed by quantitative real time PCR.

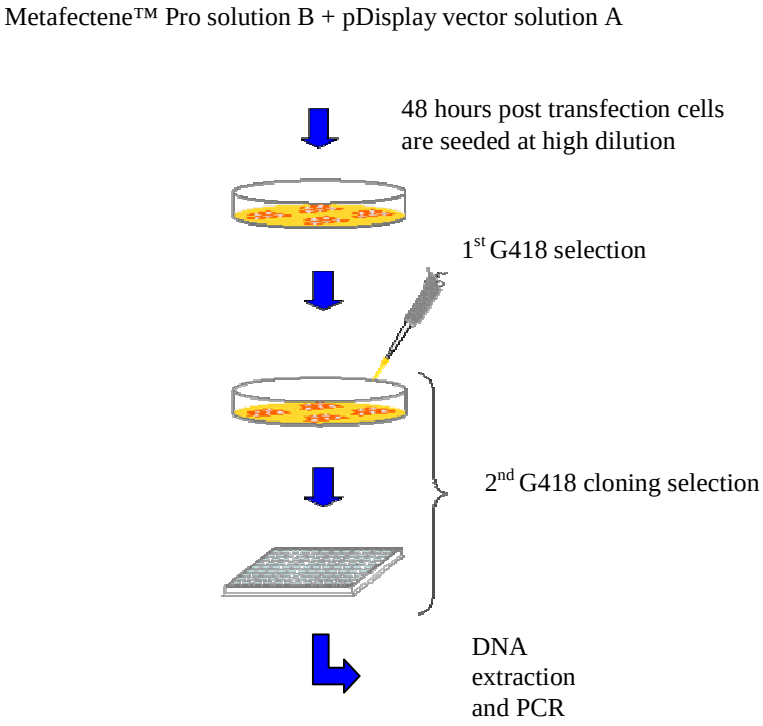


Figure 16: Procedure for antibiotic monoclonal clones selection

2.6 IMMUNOFLUORESCENCE

Immunicytochemistry was done on the transfected cells to confirm the presence of the inserted constructs and to determine their cellular localization. 20.000 cells seeded on each coverslips were fixed after 24 hours using 4% paraformaldehyde (PFA); coverslips were washed in PBS1X (final concentration NaCl 137mM, KCl 2.7mM, Na_2HPO_4 4.3mM, $KH_2PO_4$1.47mM, adjust to a final pH of 7.4) and if necessary permeabilized using 0.1% Triton; coverslips were then incubated with primary antibody anti-HA High Affinity (clone 3F10, Roche Diagnostic) used diluited 1:300 in a mixture that included 10% NGS (normal goat serum) and PBS1X and with primary antibody anti-human Glypican-5 (clone 297716, R&D Systems) used diluited 1:200 in a mixture including 10% NGS and PBS1X; after overnight incubation at 4°C, cells were incubated for an hour at room temperature with the secondary appropriate antibody in a mix with 10% NGS and PBS1X: secondary antibody against HA used was an anti-Rat Alexa 488

(Invitrogen) diluited 1:400, and the secondary antibody used to reveal GPC5 was an anti-Mouse tagged FITC or TRITC diluited 1:5000. Nuclei are revealed with incubation of coverslips with Hoechst 33258 staining and then the coverslips were mounted and observed under epifluorescence Nikon eclipse E600 microscope connected with a Nikon DXM1200 camera.

2.7 FLOW CYTOMETRY

500.000 over-expressing GPC5 cells and control cells were collected and incubated with 250ng of direct-PE labeled monoclonal antibody anti-human/mouse GPC5 (clone 297716, R&D System) on ice and then washed with PBS1X and resuspended in PBS1X for the analysis. As a control, cell in separate tube were treated with PE-labeled mouse IgG_{2A} isotype control (clone HOPC-1, SouthernBiotech) antibody. All the sample were analyzed on fluorescence activated cell sorter (Coulter EPICS XL-MCL Flow Cytometer) with the collaboration of Marco Vitale groups (Department of Anatomy, Pharmacology and Forensic Medicine, University of Parma, Italy).

2.8 WESTERN BLOTTING

Cell extracts were prepared by lysing the cell for 10 min on ice in RIPA lysis buffer (final concentration 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. Protein concentration was measured by Bradford method[134,135]. Supernatant of stable cell lines were collected after 24 hours of culture incubation in DMEM serum free and concentrated utilizing methanol protocol[136]: 400 µl of supernatants were mixed with 2.5 volumes of cool 100% methanol, incubated overnight at -20°C, washed the precipitate with cool 100% acetone, the pellet recovered were dissolved 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%). Protein extract diluited in 2X sample buffer and concentrated media were resolved on 8% SDS-PAGE and transferred to Hybond-P membranes (Amersham Pharmacia) over day. Western blot analysis were performed using the following primary antibodies: anti-HA High Affinity (3F10, Roche Diagnostics) used 1:2000 in TBS with 0.1% Tween® and 5% skim milk, anti-human Glypican-5 (R&D) used 1:500 in TBS with 0.1% Tween®, anti-actin (Sigma Aldrich) used 1:3000 in TBS with 0.1% Tween® and 1% skim milk as control. After incubation with the primary antibody, the blots were washed and incubated for 1 hour with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody; labeled proteins were detected with an ECL-Plus detection system (Amersham Pharmacia).

2.9 PROLIFERATION ASSAY

Viable cell numbers were estimated by the Trypan blue exclusion test. Cells were seeded in triplicate in 6-well culture plates at a density of $1x10^5$ cells/well in DMEM with 10% and 1% of FBS in two different experiments. At various time points, 24, 48, 72 hours, cells in individual monolayer cultures were made into single-cell suspensions by trypsinization. The cell suspensions were then incubated for 3 min with Trypan blue dye and counted using a Burker chamber (Beckman Coulter-Z2). The cells remaining negative to the blue dye staining after Trypan blue exclusion were counted as viable cells.

2.10 CELL ADHESION ASSAYS

The adhesion assays were performed by using different purified ECM molecules: Collagen type III, Collagen type V, Laminin-1, Fibronectin and Vitronectin. The coverslips were coated using a 20 μg/ml of each matrix (Fibronectin, Laminin-1 and Vitronectin diluted in PBS1X and Collagens diluted in acetic acid 0.02N). The coatings

were done for 2 hours at 37°C for Fibronectin and at room temperature for the other molecules. Post-coating, $4x10^4$ cell/ml cells were seeded on the coverslips coated in the same medium of the culture DMEM with 1% of FBS, as specified in proliferation assay. After 30 minutes from cells seeding, the cells were fixed in 4% PFA and if necessary permeabilized using 0.1% Triton; after the fixation the cells were subjected to immunocytochemistry and coverslips were incubated with primary antibody anti-HA (in the same condition previously reported) and primary antibody against actin, Phalloidin-TRITC (Phalloidin has a specific binding for F-actin) used diluited 1:300 in PBS1X at room temperature; nuclei were stained as previously reported. Coverslips were mounted and observed under epifluorescence Nikon eclipse E600 microscope connected with a Nikon DXM1200 camera.

2.11 CELL MIGRATION ASSAYS

To examine haptotactic migratory behavior of GPC5 over-expressing cells on the same panel of isolated ECM molecules utilized in adhesion assay, it was performed a classical Transwell assay whereas to examine how GPC5 influenced the overall locomotory abilities of the cells it was used a conventional scratch assay.

These assays were done using 8-µm pore FALCON HTS FluoroBlock Transwell system (Becton Dickinson) (Figure 17). The inserts of the Transwell were coated with the panel of different purified ECM molecules in the same condition showed in the adhesion assays. Cells were stained with Cell Tracker ORANGE (C2925 Invitrogen) and seeded into the insert at concentration of $1x10^5$ cell/ml/transwell in DMEM with 1% of FBS; the lower chamber contained DMEM 10%. After 16 hours from cells seeding it was valued the number of migrated cells through the matrix component using the Inverted Nikon Eclipse TS 100 fluorescent microscope.

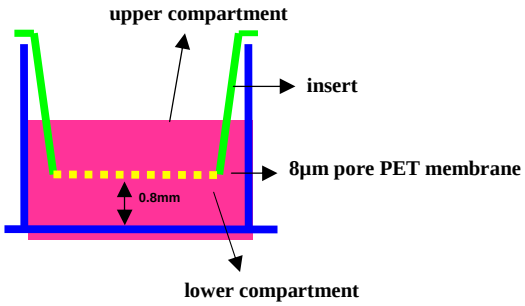


Figure 17: FALCON HTS FluoroBlock Transwell system.

Wound-healing-type (scratch) assays were performed by using Ibidi Culture System (80206 Ibidi, BioDiagnostic). The silicon insert creates 2 reservoirs separated by a 400µm thick well. Cells were seeded into each well at concentration of $3x10^5$ cell/ml in DMEM with 1% of FBS and were incubated at 37°C and 5% CO_2. After 24 hours the cells reached confluence and the insert was removed by using sterile tweezers from the surface resulting in 2 defined cell patches separated by the original strip of 400µm (Figure 18). The used well were filled with DMEM with 1% of SFB. From this moment it was began the monitoring of the cell migration kinetic using Inverted Nikon Eclipse TS 100 fluorescent microscope in phase contrast and were done microscope images at established time: 0 hours (start time), 3 hours, 5 hours, 7 hours and 24 hours (stop time) and were measured how much the front of migratory cells was moved from the start point during the time of observation with Adobe Photoshop program converting pixel of microscope images in µm.

a **b**

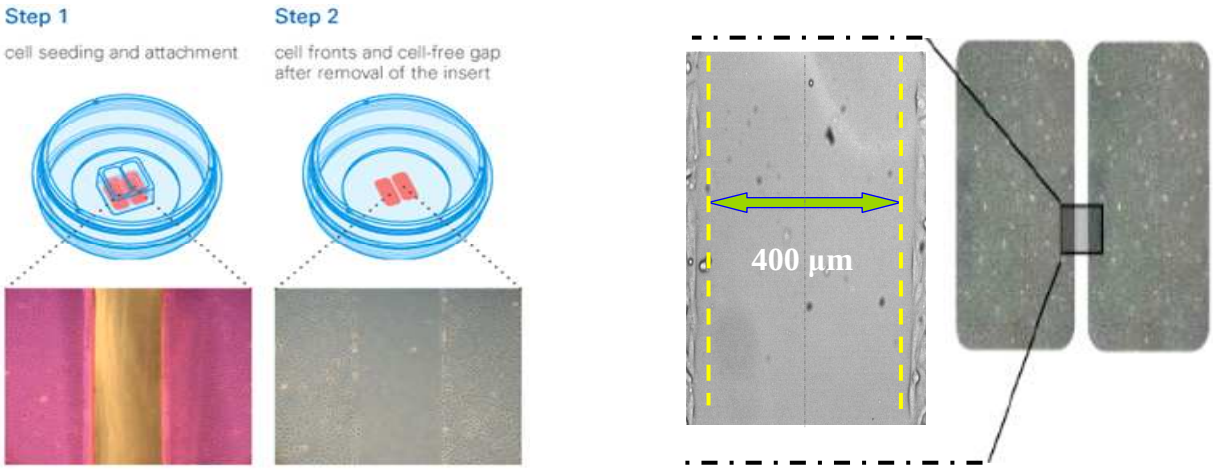


Figure 18: a) Steps of Wound healing assay using Ibidi Culture System; b) field of interest considered to take microscope images (magnification 10X).

2.12 STATISTICAL ANALYSES

In all experiments of adhesion and migration was applied as statistical test the *Student's t*-test to determine the statistical significance of differences between means of three independent experiments, each in triplicate. Significance was defined as a $P<0.05$.

3. RESULTS

3.1 DEFINITION OF THE CONSTITUTIVE PROTEOGLYCAN SURFACE PATTERN IN SARCOMA CELLS AND OSCC BIOPSIES

A first part of the work was dedicated to the definition of the constitutive pattern of NG2, glypicans and syndecans expression in 31 of the sarcoma cells listed in (Table 9) at the transcriptional level. The results of this initial screening by qualitative PCR are reported in Table 12.

Table 12: Expression pattern of PGs in sarcoma cells subdivided by hystotype.

	NG2	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-MS-1				+	+	+	+	+	+	+	+

	NG2	GPC1	GPC2	GPC3	GPC4	GPC5	GPC6	SDC1	SDC2	SDC3	SDC4
NTI-MFH-1	nd	+					+	+		+	
NTI-MFH-3	nd	+					+			+	+
NTI-MFH-4	+							+	+		+
143B	+	+	+		+	+	+	+	+	+	+
MG63	+							+	+		+
SAOS2	+						+		+	+	+
NTI-OS-1	+		+	+	+	+	+	+	+	+	+
RD	+			+	+			+	+	+	+
A204	nd	+					+	+		+	+
SJRH30	+	+	+	+	+	+	+	+	+	+	+
SW982	(+)	+				+	+	+		+	+
NTI-PNS-1	nd	+				+	+	+		+	+

[1] nd: not determined

The panel of sarcoma cells analyzed represents several hystotypes of which six are correspondent by more than one cell. When looking at how PGs are distributed in this cells, it is evident that GPC2 and GPC3 are less frequently expressed (only in three distinct hystotypes), whereas SDC3 and SDC4 are the PG more widely expressed. GPC1, SDC1, SDC3 and SDC4 are expressed in all fibrosarcoma cells, whereas other PGs are differently expressed. In leiomyosarcoma cells, SDC3 and SDC4 are always present and GPC1 and SDC1 are expressed in all cells, with the exception of NTI-LMS-1 and NTI-LMS-6; in three of seven leiomyosarcoma (NTI-LMS-5, -6 and -8) GPC4 and SDC2 are expressed and other PGs are differently present. All liposarcoma cells are similar in PG expression pattern except for NTI-LS-2, which expresses only SDC2. In the three malignant fibrohistiocytoma cells, seems to be at random PGs expression. In osteosarcoma cells there is a constant expression of NG2, SDC2 and SDC4 and two of them (143B and NTI-OS-1) express 10 PGs on the total of 11 with the expression of the

not frequent GPC2, whereas it is to note that NTI-OS-1 expresses also GPC3. All rhabdomyosarcoma cells always express SDC1, SDC2 and SDC4; RD and SJRH30 cells also express NG2, GPC3, GPC4 and SDC2. Finally, to note is that GPC3 is also expressed in the only Mullerian sarcoma cells analyzed.

To the same aim to define the qualitative constitutive pattern of PG expression, we have analyzed 22 sample obtained from surgical specimens from patients with OSCC as previously reported (Table 8) subdivided in 4 classification groups that are been defined follow the sequent characteristics: patients in stage T1-T2 that show poor prognosis within or more than 40 years old and patients affected to advanced OSCC (T3-T4) with a good prognosis within or more than 40 years old (Table 13) and the results are reported in Table 14. Only two groups (sample TNM classified as T1T2 and age major of 40 years and sample TNM classified as T3T4 with age major of 40 years) are potentially informative, because the other two groups (sample TNM classified as T1T2 and age minor of 40 years and sample TNM classified as T3T4 with age minor of 40 years) are constituted at most of 2 samples; besides, 4 sample (19, 20, 21 and 22) were excluded from classification because are lacking of essential information (TNM classification and/or age).

Table 13: Grouping of samples derived from surgical specimens and subdivided in relation to their TNM classification and age of patients**.**

Classification groups		**N° sample**
I	T1T2 < 40 yrs	2
II	T1T2 > 40 yrs	7
III	T3T4 < 40 yrs	1
IV	T3T4 > 40 yrs	8
V	excluded	4
	total	22

Table 14: Expression pattern of PGs in surgical specimens subdivided in different classification groups

		CSPG4	GPC1	GPC2	GPC3	GPC4	GPC5	GPC6	SDC1	SDC2	SDC3	SDC4
I												
	4		+					+	+	+		+
	6							+	+	+	+	
II												
	7		+				+	+	+	+	+	+
	9							+		+	+	
	10							+	+	+		+
	12		+						+	+	+	+
	14	+						+	+	+	+	+
	15		+		+	+		+	+	+		+
	16		+	+				+	+	+	+	+
III												
	2							+	+			+
IV												
	1											
	3		+					+	+	+	+	+
	5	+	+			+	+	+	+	+	+	+
	8	+			+			+	+	+	+	+
	11		+		+	+		+	+	+	+	+
	13		+		+			+	+	+		+
	17		+					+	+	+	+	+
	18							+	+	+		+
V												
	19					+		+	+	+		+
	20							+	+	+		+
	21		+			+	+	+	+			+
	22		+						+	+	+	+

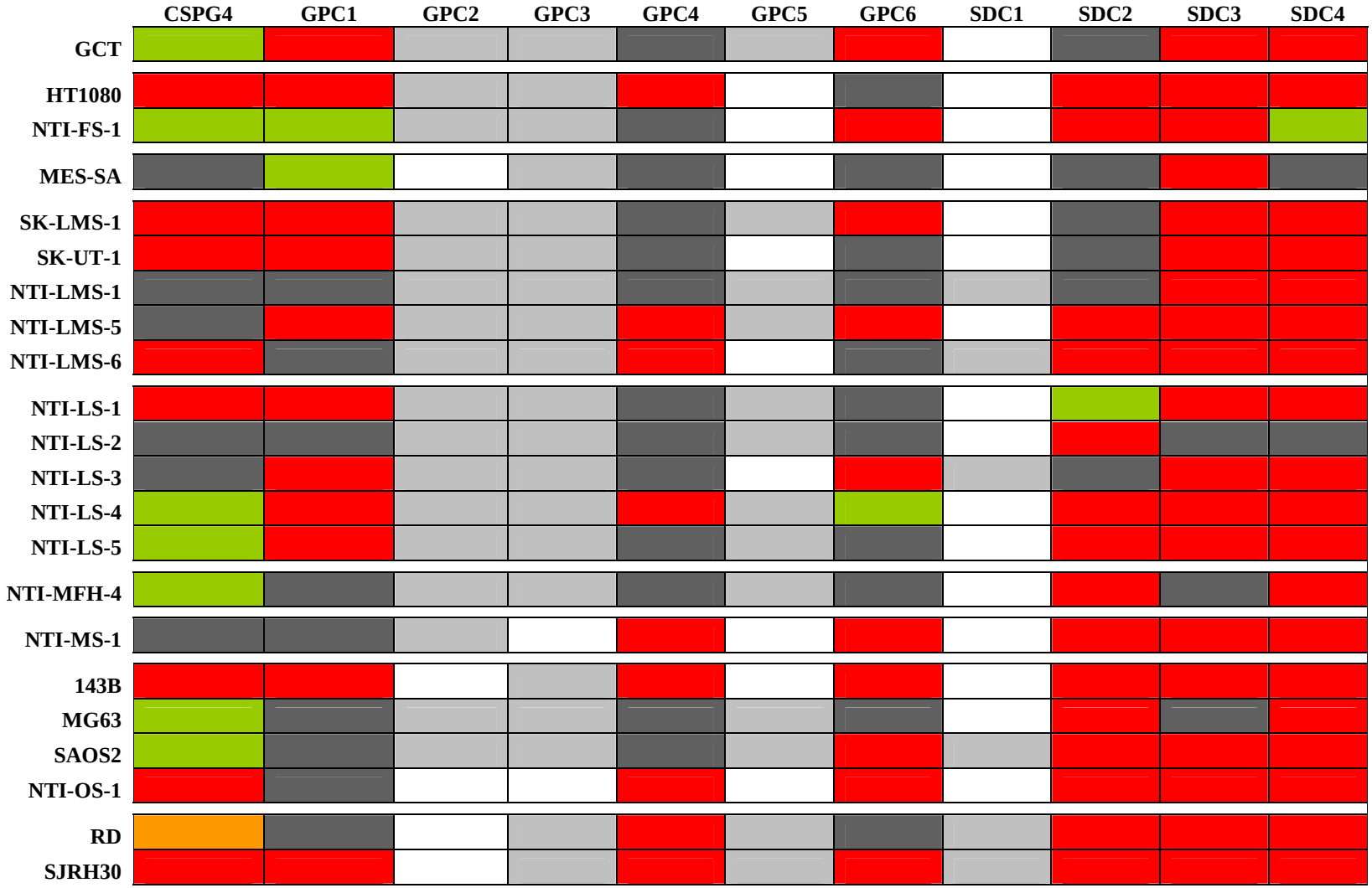
In a first analysis shown in Table 14 for the two groups in which there was a sufficient amount of cases, II and IV, we can observe that in II there is a constant expression of

SDC2 and 7 sample on a total of 8 express GPC6, SDC1, SDC3 and SDC4; GPC1 is expressed in the 50% of samples and there is a rare expression of NG2, GPC2, GPC3 GPC4 and GPC5. In IV, with the exception of sample 1, that are lacking of the expression of all PGs, all of others samples express GPC6, SDC1, SDC2 and SDC4; five on a total of 7 sample express GPC1 and SDC3 and there is only one sample that express GPC5 and none express GPC2. It can be observed that in IV, GPC3 is expressed in three samples.

To define more in detail the PG expression profile in both sarcoma cells and biopsies of OSCC it is essential to analyze the comparative PG expression in order to understand if different levels of expression of the same gene in various sarcoma hystotypes or classification groups in patients with OSCC is an informative element.

Expression of NG2, glypicans and syndecans of sarcoma cells were normalized to endogenous housekeeping gene 18S rRNA and it was chosen cDNA of human mesenchymal stem cells (hMSC) as sample calibrator, because mesenchymal cell is the ancestral common cell of all sarcomas. The results of over-expression or down-regulation of PGs expression level are reported in Figure 19. In Figure 20 are reported the level of relative PG expression for sarcoma cells grouped in hystotypes only for which there are more than two sarcoma cells analyzed.

Figure 19 (next page): TaqMan low-density arrays results: gene expression level down-regulate , up-regulate or unchanged in comparison to hMSC as sample calibrator; ddd indicate that the cells not express the gene, indicate that the gene is not expressed in both cells and sample calibrator, indicate that are not possible to calculate relative expression.


CSPG4
GPC1
GPC2
GPC3
GPC4
GPC5
GPC6
SDC1
SDC2
SDC3
SDC4
GCT
HT1080
NTI-FS-1
MES-SA
SK-LMS-1
SK-UT-1
NTI-LMS-1
NTI-LMS-5
NTI-LMS-6
NTI-LS-1
NTI-LS-2
NTI-LS-3
NTI-LS-4
NTI-LS-5
NTI-MFH-4
NTI-MS-1
143B
MG63
SAOS2
NTI-OS-1
RD
SJRH30

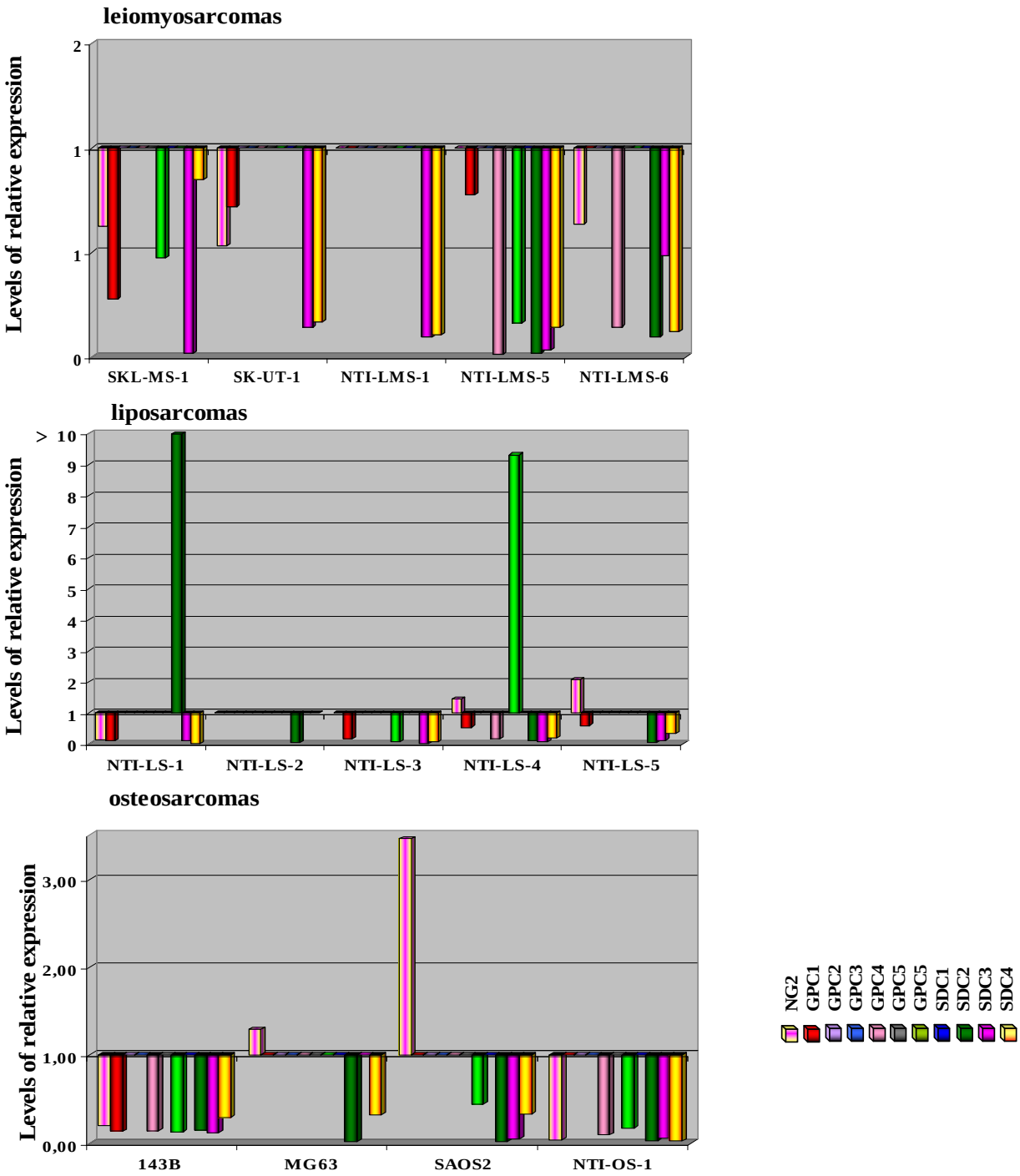


Figure 20: Level of relative expression of PGs of our interest in sarcoma cells subdivided in hystotypes. Data are normalized for the expression levels with hMSC, used as sample calibrator.

Results of qPCR show that in leiomyosarcoma cells all PG expressed are down-regulate and where there is the expression of NG2, mRNA level is 50% less than in the sample calibrator. In liposarcomas, PGs down-regulate have a very few level of expression, whereas NG2 expression is up-regulated twofold in two cells, in NTI-LS-4 GPC6 is up-regulated more than 9 folds and in NTI-LS-1 SDC2 is expresses 96 folds in comparison to level of expression in hMSC. In osteosarcoma cells all PGs are down regulate with the exception of NG2 that is up-regulate in MG63 and SAOS2 of respectively 1 and more than 3 fold in comparison to sample calibrator.

Expression of NG2, glypicans and syndecans in biopsies was normalized to endogenous housekeeping gene 18S rRNA and it was chosen cDNA of pool of RNAs extracted from 5 normal oral epithelia surgical specimens as sample calibrator**,** because all oral squamous cell carcinomas developed from mucosal epithelial cells. The results of PG expression levels are reported in Figure 21 where it is indicate that for some PGs was not possible to calculate the expression. In Figure 22 are reported the level of relative PG expression for patients divided in classification groups (Table 13) only for which there are at last two biopsies analyzed. Results of SDC1, SDC2 and SDC4 expression levels show that these PGs are up-regulate in most of the biopsies analyzed and only in few sample are down-regulate. The classification group I shows results not interpretable because two samples are few for analyses. In II all the samples have a similar pattern of expression, except for patients 10 that has SDC1 and SDC4 very few down-regulate in comparison to sample calibrator; SDC2 is the PG more express than others with a mean more than 20 folds in comparison to normal mucosal epithelium. Classification group IV show SDC1 expression level fluctuate nearly to the same level of the sample calibrator; the other two PGs, SDC2 and SDC4 are over-expressed in 85% of the samples and their expression levels in changeable within the group. In the biopsies of patients not classifiable, is interesting that one sample (20) is lacking of 80% of PGs expressions, other two (19 and 21) have a variable PG expression levels and the last biopsy (22) express 45% of PGs and all the PGs with expression level values are up-regulated more than 10 folds (SDC2 45 folds and SDC4 30 folds) in comparison to normal sample calibrator).

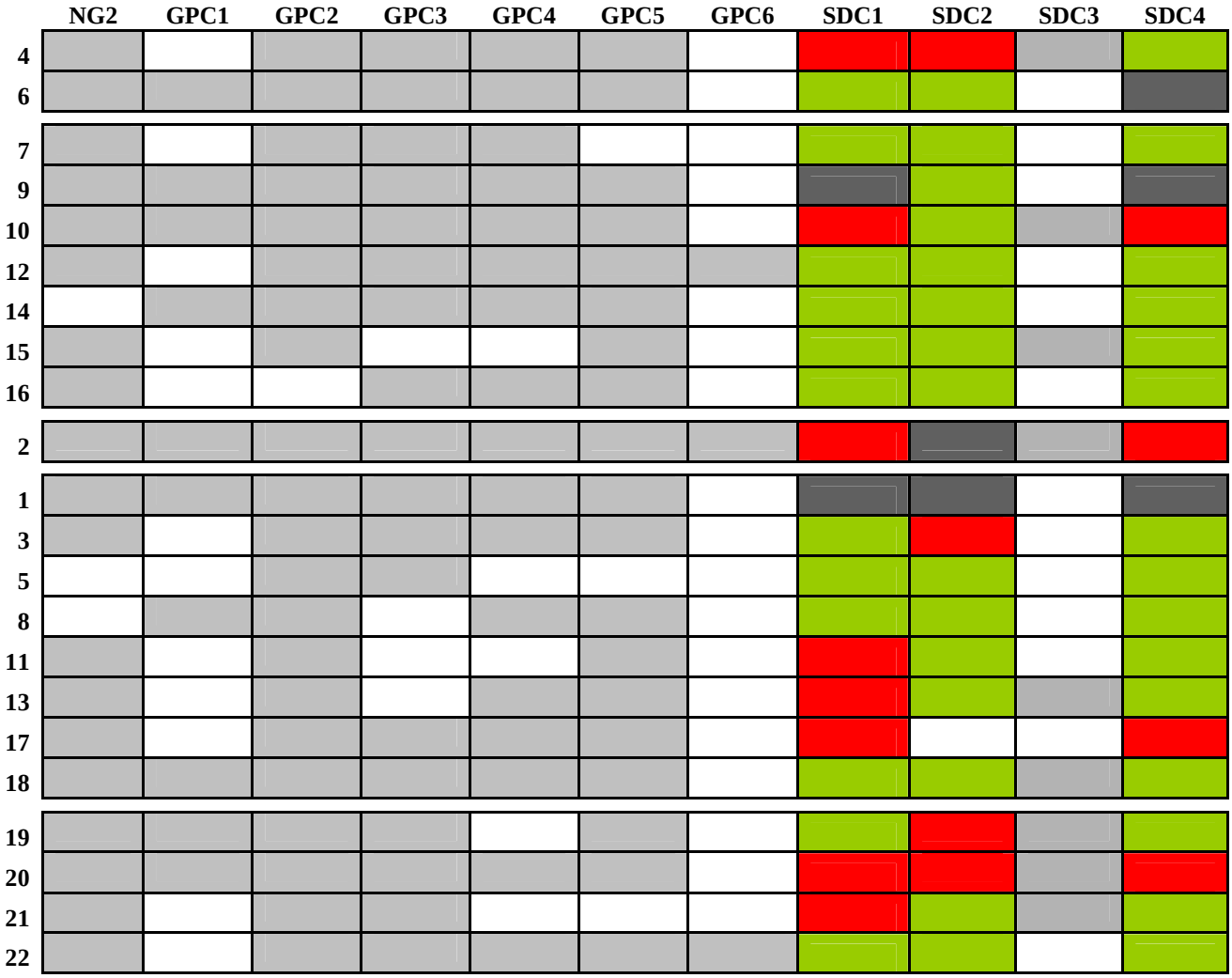


Figure 21: TaqMan low-density arrays results: gene expression level down-regulate , up-regulate in comparison to pool of RNA obtained from normal mucosal epithelium as sample calibrator; ddd indicate that the biopsy not express the gene, indicate that the gene is not expressed in both biopsy and sample calibrator, indicate that are not possible to calculate relative expression.

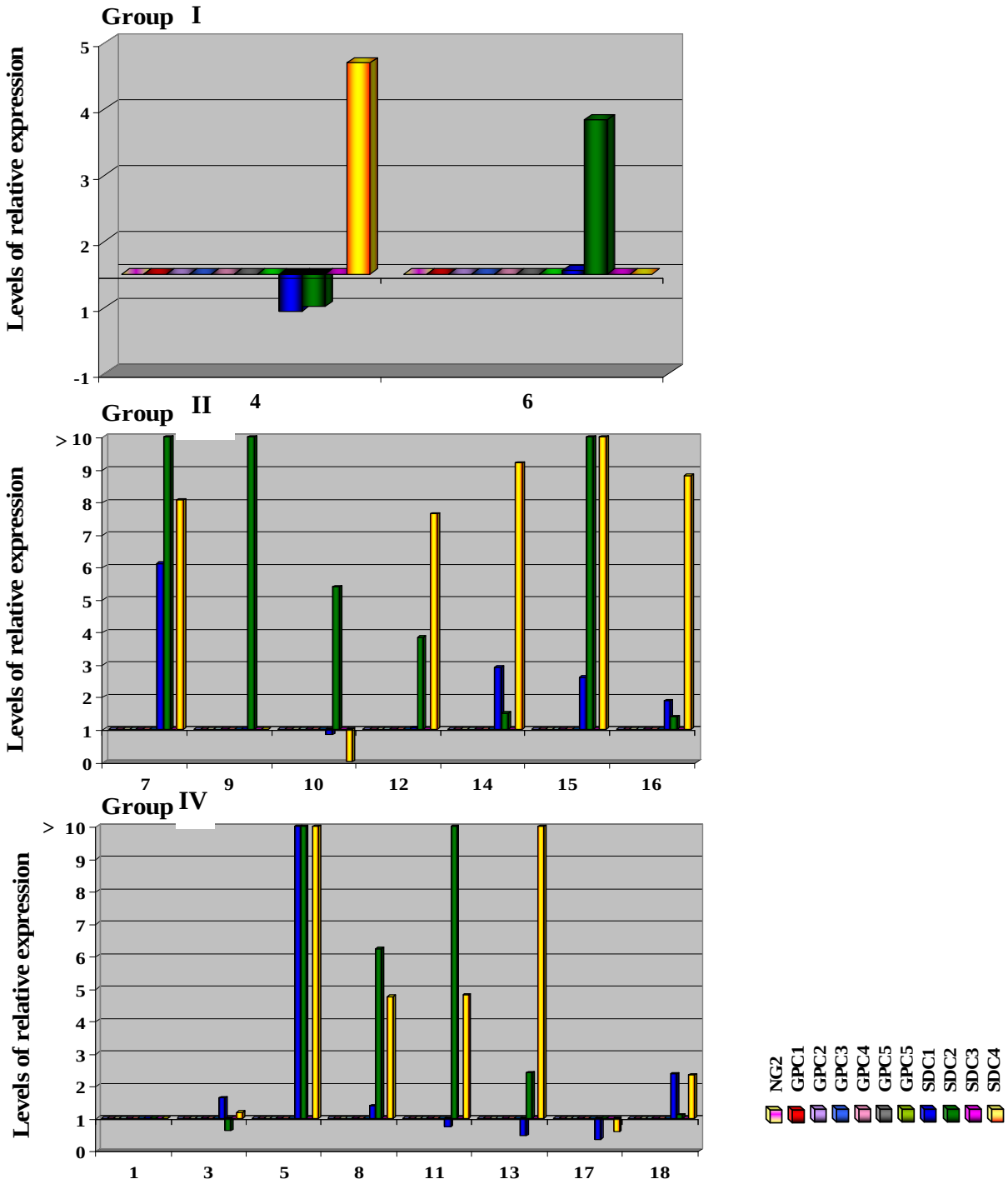


Figure 22: Level of relative expression of PGs in biopsies of OSCC subdivided into different classification groups I, II and IV. Data are normalized for the expression levels with normal oral epithelia used as sample calibrator.

Combining the result of PG expression obtained by RT-PCR and by TaqMan Low Density Array, we could define 24 different cellular PG models at the transcriptional level (Figure 23) with discrete surface PG pattern available for subsequent molecular manipulation, as gene transduction. Each cellular model can be in common between two or more sarcoma cell even if they belong to different hystotype.

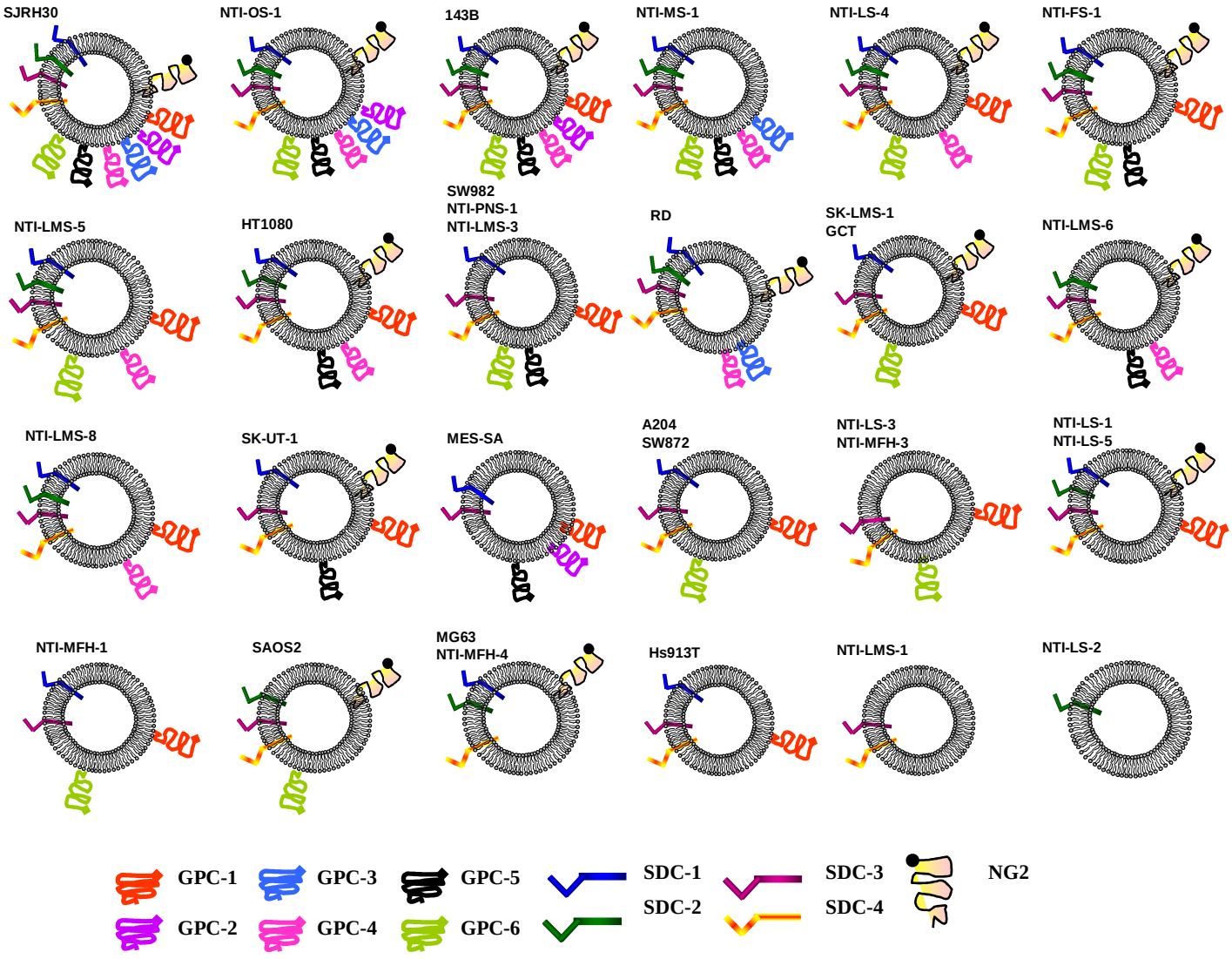


Figure 23: Cellular model available; two or more cells of different hystotype can be having the same pattern of PGs.

3.2 OVER-EXPRESSION OF GLYPICAN-5 IN 143B CELLS

We have decided to analyze more in detail the function of glypican-5 because there is little information about its role in tumour progression and especially in sarcomas (as already discussed in the Introduction). By taking advantage of the PG patterns previously defined, we have chosen 143B cells to start to over-express GPC5 because this cells has a rather complex pattern of PGs expression that can allow us to combine different techniques, as in vitro transduction and/or RNAi mediated knockdown of individual cell surface PG in relation to their constitutive level of expression.

We generated 5 stable clones of 143B cells over-expressing GPC5 ($GPC5^+$ cells) with two of them over-expressing the gene at high level. We have chosen to use in *in vitro* assays one of the two clones with more than 90% of the cells over-expressing glypican-5 as detected by Western blotting (Figure 24), immunocytochemistry and flow cytometry (Figure 25). The levels of expression of GPC5 mRNA in $GPC5^+$ cells was assessed by qPCR and compared to control cells (CTR cells).

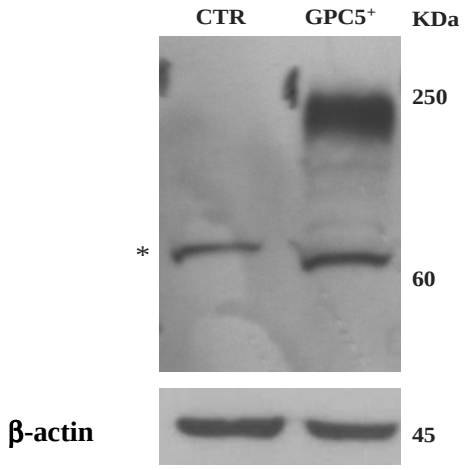


Figure 24: Western blotting to detect the presence of glypican-5 on $GPC5^+$ cells;* indicate a nonspecific band.

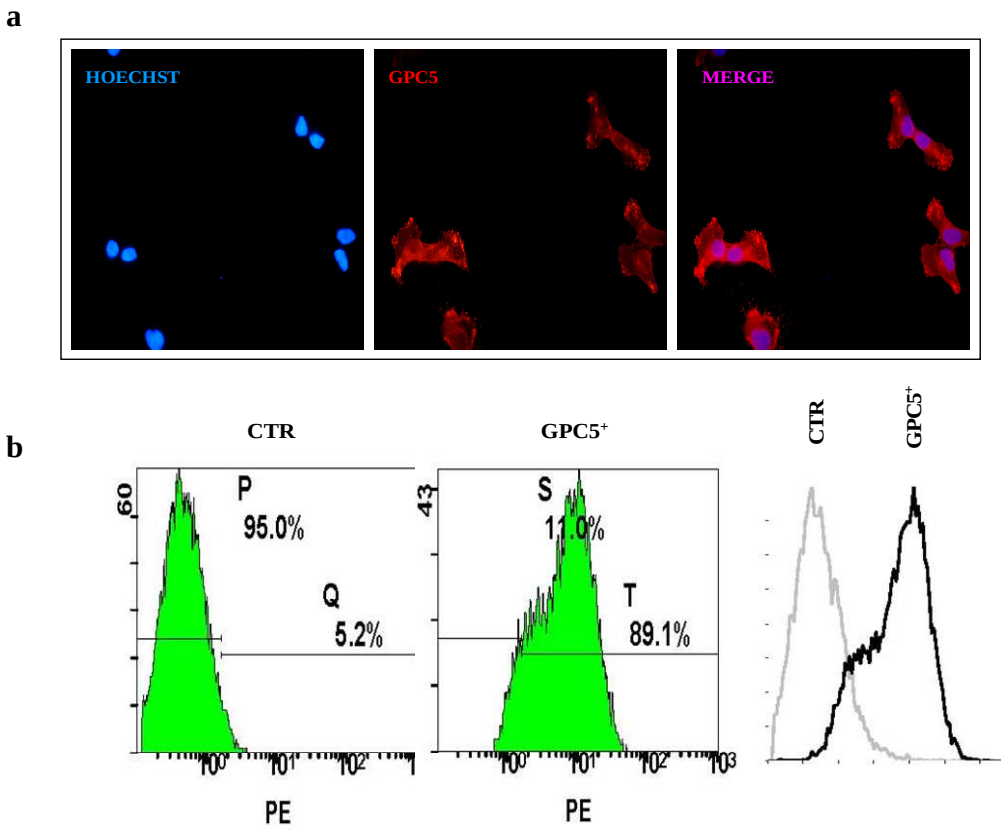
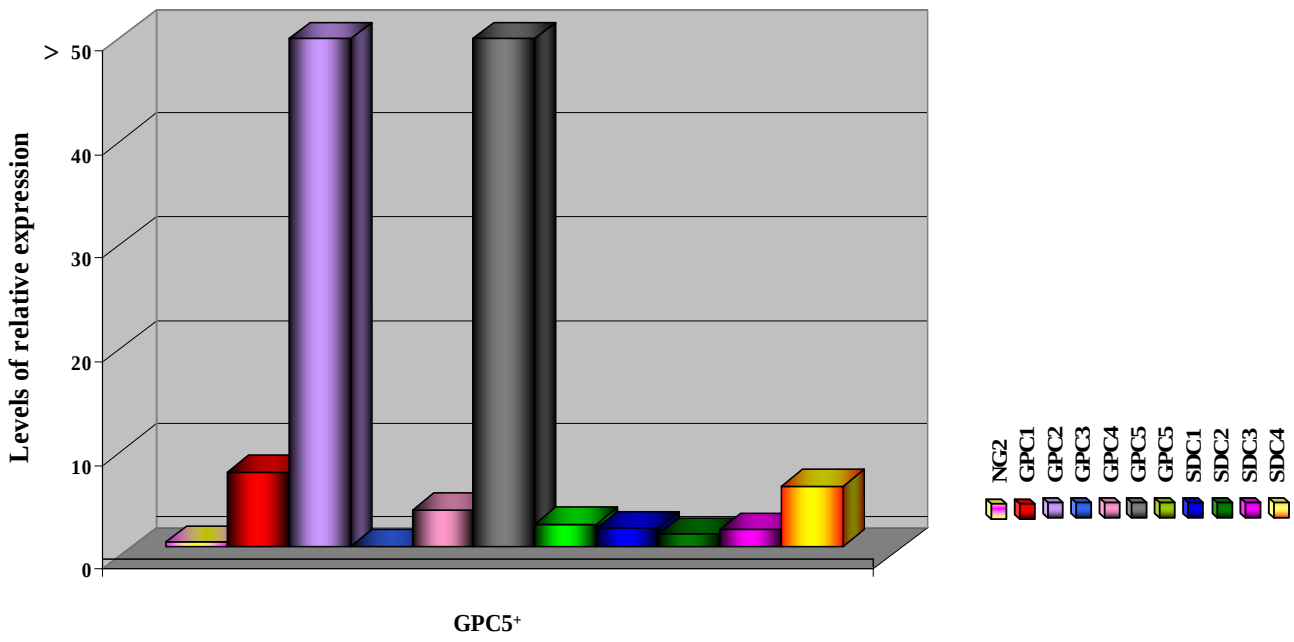


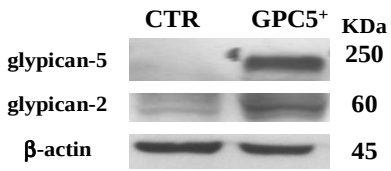
Figure 25: Detection of GPC5 in over-expressing cells by a) immunofluorescence (magnification 60X) and b) flow cytometry.

Since in some pilot experiments we have noted that manipulation of given surface PGs frequently alters the relative expression of others, we evaluated how transduction of GPC5 affected the expression levels of the others PGs present on GPC5$^+$ cells in comparison to CTR cells (Figure 26a). It is evident from the graph of levels of relative expression of the other PGs on the cell surface of stable GPC5$^+$ cells that the over-expression of GPC5 modulate almost all the expression of the other PGs and in detail the expression level of GPC1 and SDC4 is increased more than 5 folds and GPC2 is up-regulated of 80 folds in comparison to the expression in the control cells and in Western blotting assay is confirmed this expression profile for glypican-2 with the result of the creation of two new and different cellular model (Figure 26b,c).

a



b



c

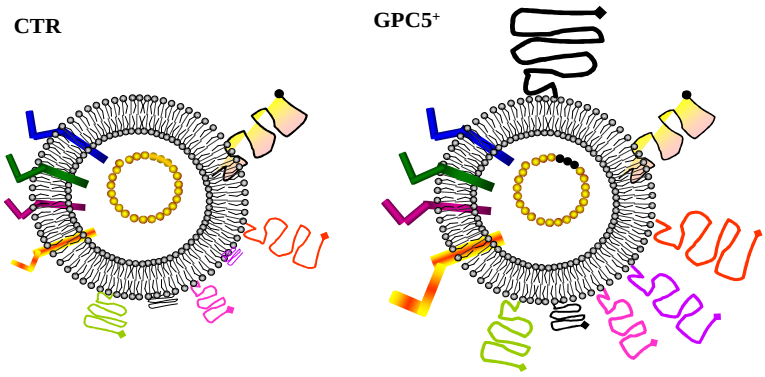


Figure 26: a) Levels of relative expression of PGs in GPC5 over-expressing cells (GPC5$^+$ cells) in comparison to control cells (CTR cells); b) Western blotting of over-expression of glypican-2 in GPC5$^+$ cells and c) CTR and GPC5$^+$ new cellular model.

3.3 ANALYSIS OF POST-TRANSATIONAL MODIFICATIONS OF GLYPICAN-5

In order to understand if glypican-5 could be shedded, as previously described for glypican-1 and glypican-3, from the cell surface by proteases/lipases-mediated cleavage of the glycosyl-phosphatidylinositol (GPI) anchor, a Western blotting analysis was performed in search of the N-terminal domain of the glypican-5 in the medium of cultured GPC5$^+$ cells after 12 hours of starvation in serum free medium (Figure 27).

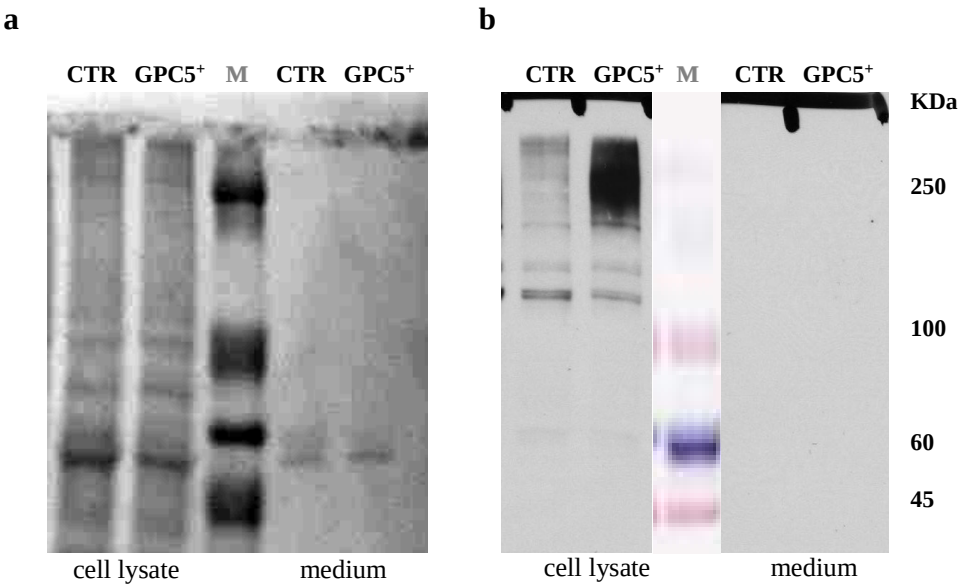


Figure 27: Assessment of glypican-5 released by GPC5$^+$ cells into the culture medium by cell surface shedding: a) Ponceau staining of a representative SDS gel; b) Western blotting to detect the presence of glypican-5 on GPC5$^+$ cell lysate (as control) and on GPC5$^+$culture medium; M is the protein ladder ColorBurst® (SIGMA).

In this first experiment aimed at revealing the presence of glypican-5 in the culture medium of GPC5$^+$ cells, it was not possible to detect the N-terminal portion of the PG; this suggested that there was little or no surface release of the transduced glypican-5 by enzyme-mediated shedding.

3.4 EFFECT OF GPC5 OVER-EXPRESSION ON CELL PROLIFERATION

Two cellular models analyzed show similar proliferation and survival rates in optimal growth conditions (Figure 28) and in specific when cells are maintained in DMEM with 1% of SFB and in the range of time within 24hours, and this results allows us to perform *in vitro* assay of adhesion and migration without the possibility that the different cellular vitality and proliferation can influence the results.

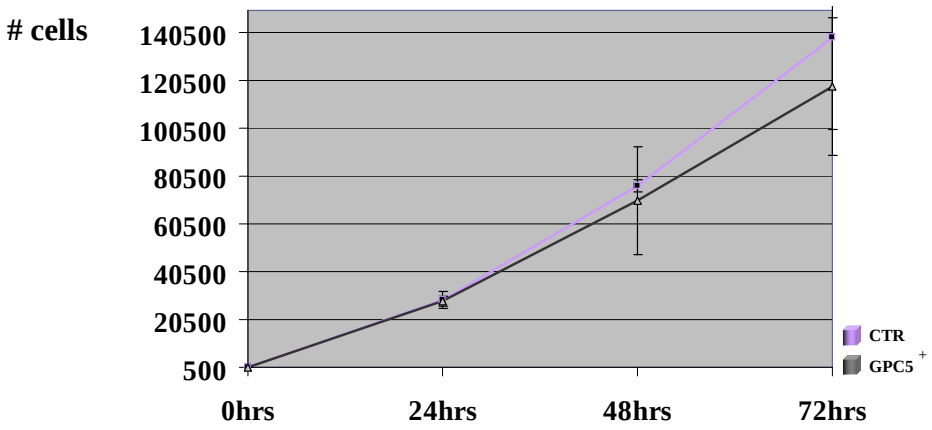
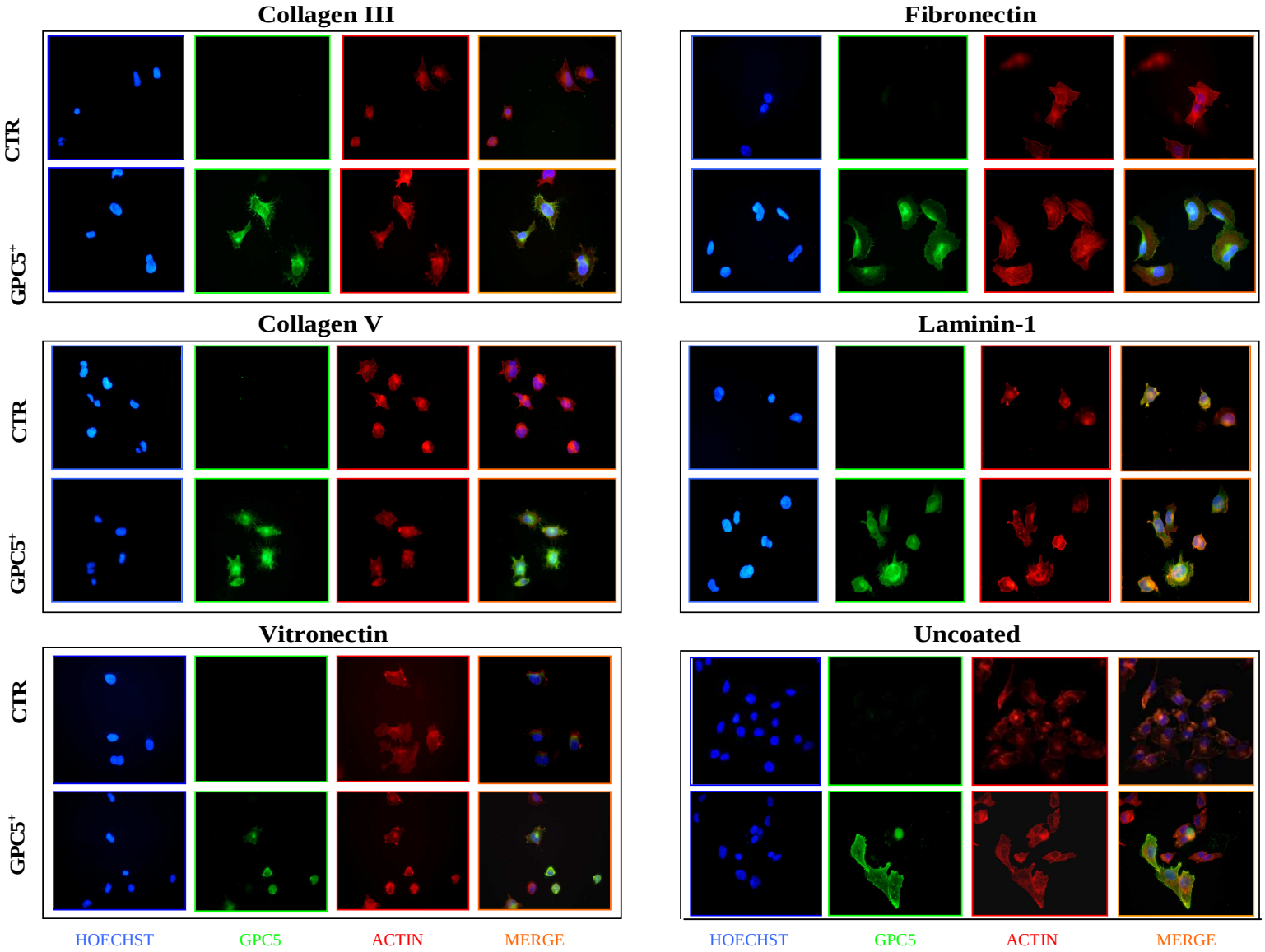


Figure 28: Growth curve of GPC5^{+} cells and CTR cells under optimal growth condition

3.5 RESPONSE OF GPC5^{+} CELLS TO ECM

We have examined the behavior of GPC5^{+} cells confronted with a first panel of isolated ECM molecules, including of Collagen type III and V, Laminin-1, Fibronectin and Vitronectin (Figure 29). Staining of cells for actin microfilaments reveals that over-expression of GPC5 and the PG pattern associated with this over-expression could be implicated in the rearrangement of the cytoskeleton, especially when GPC5^{+} cells were seeded on Collagen type III, Fibronectin and Laminin-1, whereas glypican-5 not does seems to influence the formation of stress fibers when cells were allowed to adhere to other ECM molecules.

Figure 29 (next page): Overview of GPC5^{+} adhesion on a selected panel of isolated ECM molecules (magnification 60X).


Collagen III
Fibronectin
Collagen V
Laminin-1
Vitronectin
Uncoated
CTR
GPC5+
HOECHST
GPC5
ACTIN
MERGE

GPC5^{+} cells adhered to and spread more tenaciously on Laminin-1 and Vitronectin (Figure 30); in fact GPC5^{+} cells spread 15% more than the CTR on Laminin-1 and almost twofold on Vitronectin.

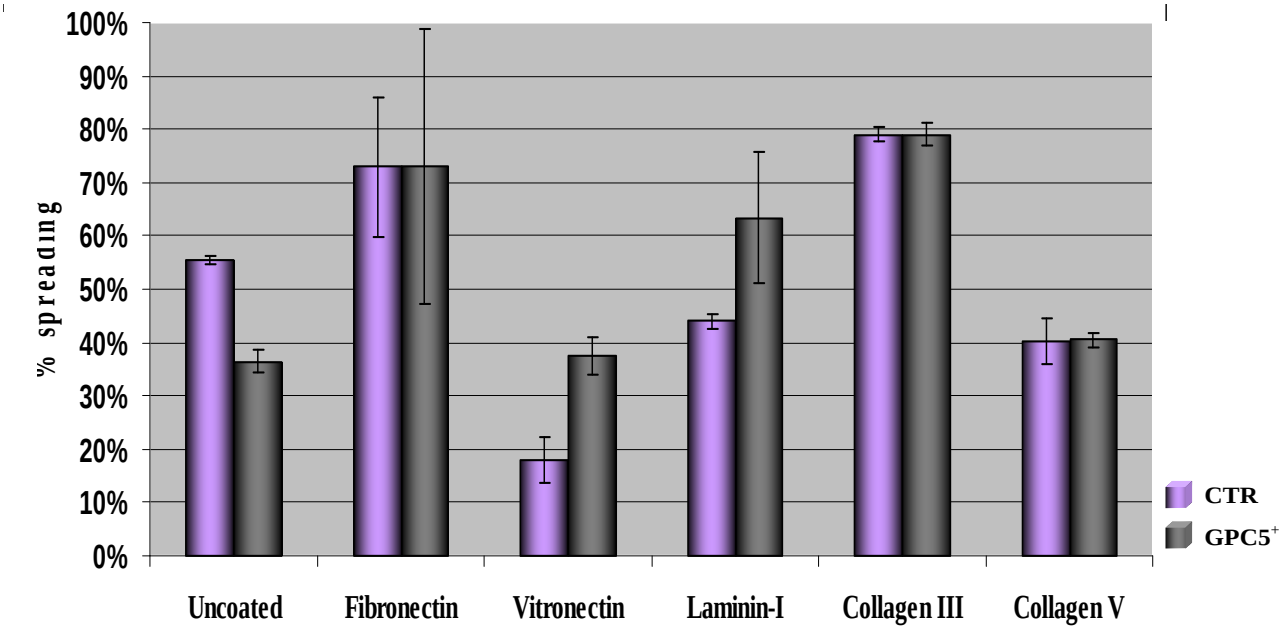


Figure 30: Ability of GPC5^{+} to adhere and spread on 5 different component of ECM

The relationship between over-expression of glypican-5 and the migration capacity of the cells on the same ECM molecules was assessed using a haptotactic migration assay, and it were found that, somewhat differently than what was seen in the spreading assay, cells over-expressing glypican-5 migrate more pronouncedly on Vitronectin and Collagen type III and less efficiently on Fibronectin in comparison to the behavior of control cells; and in the Figure 31 was showed that GPC5^{+} cells migrate less than 40% compared with CTR on Fibronectin and about more than 32% and 44% respectively on Vitronectin and type III Collagen. The haptotactic migration on the other matrices did not show significant difference between GPC5^{+} cells and CTR cells.

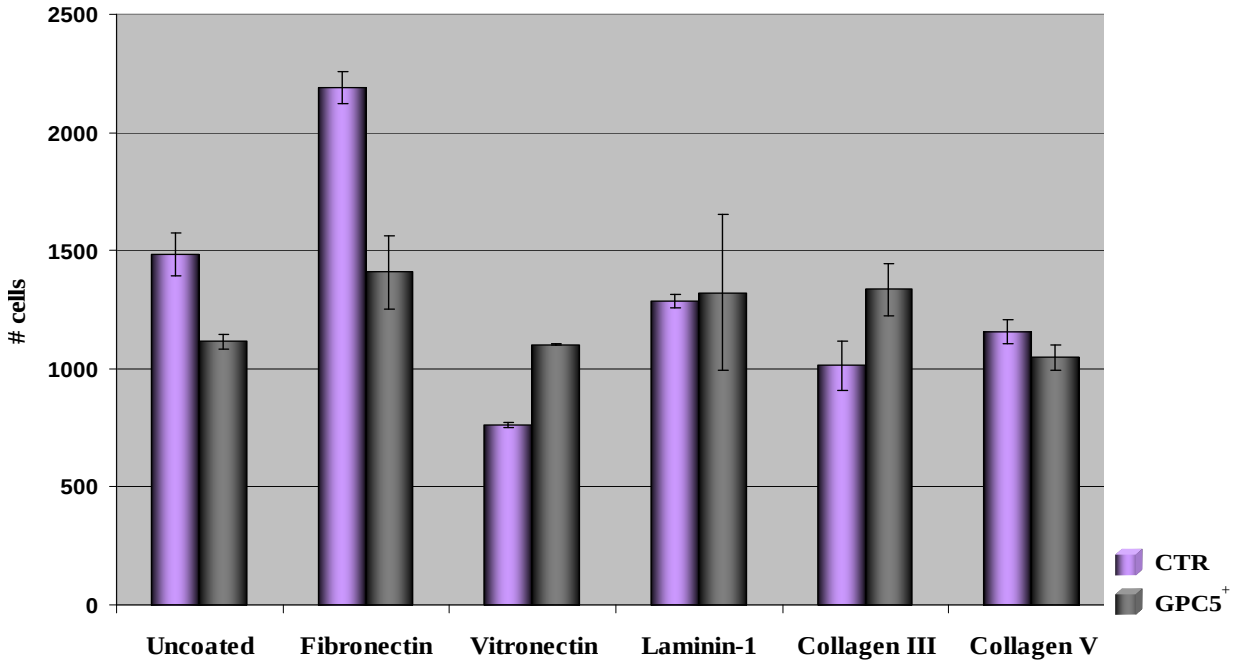


Figure 31: Haptotactic invasion assay on isolated molecules of ECM

In order to examine also the overall locomotory abilities of $GPC5^+$ cells we have used a conventional monolayer scratch injury assay and as it can be observed from the microscope images, $GPC5^+$ cells and CTR cells show a similar migratory behavior within 7 hours, then $GPC5^+$ cells begins to shorten the distance, and at 24 hours cells over-expressing glypican-5 have almost completed the coverage of the wound, while control cells have largely failed to do so (Figure 32). The analysis of results about the migration kinetic (Figure 33) points out this trend: at 7 hours $GPC5^+$ cells have covered 25% of the scratch and only 7,5% more than the CTR cells without significant difference; at 24hours glypican-5 over-expressing cells have migrated for more than 80% of the wound and 32% more than control cells.

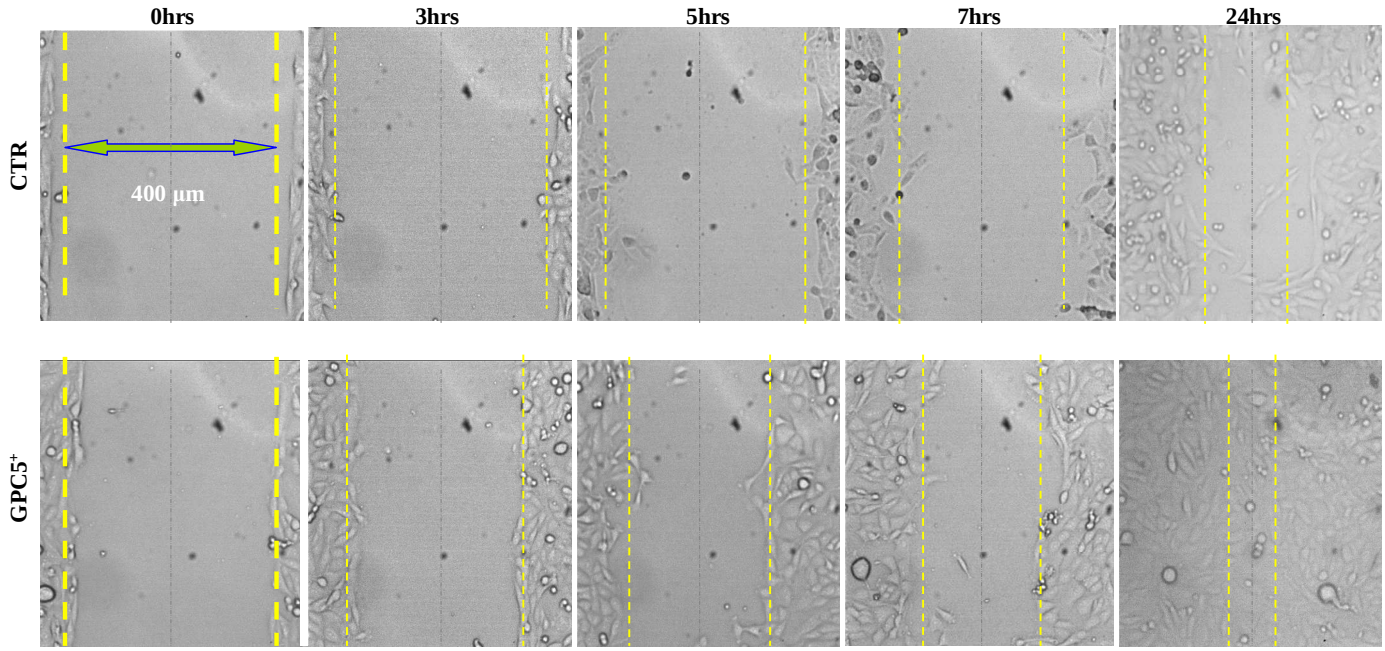


Figure 32: Determining the role of glypican-5 in locomotory abilities of 143B over-expressing GPC5 cells; dashed yellow line marks the cells' front at every time analyzed and at time 0 hours marks wound edge; dashed grey line indicate the middle of the scratch.

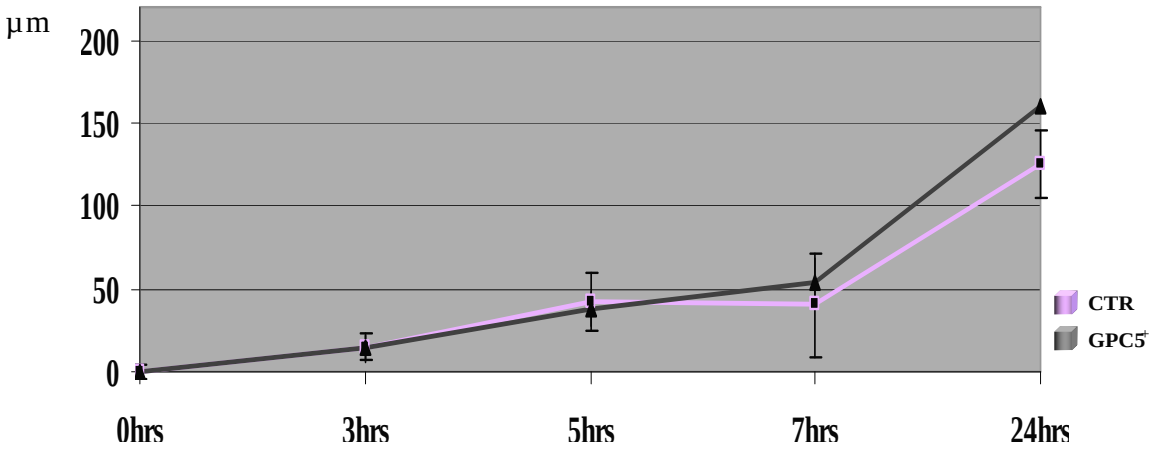


Figure 33: Migratory kinetics of GPC5^{+}

4. DISCUSSION

Accumulating evidence indicates that cellular function and phenotype are highly influenced by HSPGs, present at the cell-tissue-organ interface and they have been shown to have crucial regulatory roles in cellular control, coordinating and directing appropriate response to multiple ligands in normal physiological processes, such as embryogenesis[137], as well as in pathophysiological condition, including the processes of tumor onset and progression[138]. With respect to cancer onset and progression, the structural complexity of tumour-cell HSPGs enables them to modulate directly or indirectly several aspects of tumour-cell phenotype, including growth kinetics, invasiveness and metastatic potential[73]. So a given tumour-derived HSPG can be pro-tumorigenic or anti-tumorigenic depending on whether it is anchored at the cell surface or present in the ECM as soluble factors[139,91], as well as depending on the number and the sequences of heparan-sulphate chains attached to the core protein of HSPG and these opposing actions could be consequences of the ectopic signals produced by either their loss- or gain-of function[82]; besides; recent evidence highlights the fact that cancer cells, as part of the transformation process, alter their cell surface HSPGs profile, including differential expression of particular proteoglycans, as well as altering the HSPGs fine structure of a given proteoglycans[91,100]. In particular, glypicans, expressed predominantly during development and as HS-carrying molecules, are considered mainly regulators of heparin-binding growth factors in a tissue and cell specific manner[42] and for the reason, given the ability of glypicans to regulate the activity of growth and survival factors, reports of the last years associate changes in glypican expression with tumor progression[34,35]. In this context, we have addressed our research to understanding how different cell surface proteoglycan patterns are involved in tumor and metastasis processes especially regarding the role that different pattern of surface HSPGs could have in sarcomas development and in the identification of new prognostic/predictive markers in defined class of patients with oral squamous cell carcinomas. Since there are not data in the literature, in order to define this hypothesis, we have started to systematically define for the first time, the constitutive pattern of cell surface PGs expression in a large panel of sarcoma cells belonging to different hystotype and in biopsy of selected patients affected by OSCC. The results show that all sarcoma cells express on average of 3 to 5 cell surface PGs simultaneously and there is a widespread down-regulation of GPC1, SDC2, SDC3 and SDC4, while the

others PGs are expressed mainly hystotype specific manner and almost totally down-regulate, although from this data we are not able to define a specific profile that univocally is associated to a discrete hystotype. In fact we have outlined 24 different cellular models available and each of them could be in common with two or more sarcoma cell belonging to different hystotype. To the same aim, we have defined the cell surface PG patterns of 22 surgical specimens and the analysis of the result was carry out comparing only the two informative groups of classification (patients in stage T1T2 and T3T4 with more than 40 years old) of the 4 different determined on the basis of main characteristics that are of great importance to the classification of the tumor in oncologic patient. The data show that in the first informative groups there is a widespread but not overall over-expression of GPC6, SDC1, SDC2, SDC3 and SDC4 with rare expression of the others PGs in comparison to the second groups considered, which express in all the sample GPC6, SDC1 (over-expressed in more than 50% of samples), SDC2 and SDC4, both next to always over-expressed and GPC3 in the 37% of the specimens analyzed; both group express GPC1 in ca. 60% of samples. Remain to understand the significance of these results: the sarcoma and OSCC cell phenotype could be partly determined by surface PG expression pattern? And there are some PGs that could lead to the identification of new therapeutic target? The expression of GPC1 could regulate invasion and the response to TGFβ family of growth factors signaling[74,75,77,78]; SDC2, SDC3 and SDC4 could modulate especially the FGF2, TGFβ and Hh signalling[26,29] and be implicated in matrix assembly[24,25] and regulation of fibronectin signaling and matrix contraction together with tenascin-C[30]. Interesting is the fact that the patients of group of classification II express SDC1 and SDC2 at more high level in comparison to IV and this one express GPC3 that is not express in the normal oral epithelial and that seems similar to what happen in hepatocellular carcinoma[64,65,66,67,69,70,71,72]; it is necessary to increase the number of patients so that we will can have more PGs information to make comparison between groups of interest. In order to examine more in depth how a define PG could influences the phenotype of a sarcoma cell, we have create a new cellular model of 143B cells over-expressing glypican-5, because about the involvement of this cell surface PG in tumor, especially in sarcomas, there are very few information[90] and the osteosarcoma cell lines was a suitable model for their rather complex pattern of PG

expression. Our model has a determined cell surface PG patterns, similar to 143B parental cell lines but with over-expression of glypican-2 at high level and syndecan-4 as a results of the over-expression of GPC5, because the manipulation of a given surface PGs alters the relative expression of others as previously noted[26]. The significance of HSPGs in cell regulation is further emphasized by the presence of HS-binding domains in cell adhesion proteins of the ECM, such as Fibronectin. The concerted action of cell surface integrins and HSPGs directs cell attachment to these ECM substrates, leading to the formation of focal adhesions[12]. The capacity of HSPGs to interact with both the matrix architecture and soluble ligands defines a unique combination of properties that enables normal cells to sense, respond to and controlling influences in their environment. Cancer cells employ various mechanisms to exploit these properties and gain a survival advantage[100]. Sets out from the assumption that HSPGs on the surface of tumor cell seems to mediate their effects by regulating growth factor signaling, cell adhesion, proliferation, migration and angiogenesis[1,91,92] and because the unique structural complexity of tumour-cell HSPGs enable them to modulate directly and/or indirectly several aspects of tumour-cell phenotype, including growth kinetics, invasiveness and metastatic potential[75,76], we have decided to analyze 143B over-expressing glypican-5 cellular model for its ability to adhere and migrate in comparison to a control cells and in response to different matrix molecules in vitro, because data in literature suggest that a specific pattern of a cell surface HSPGs can be pro-tumorigenic or anti-tumorigenic depending on whether molecules of ECM can binds the GAGs chain of HSPGs or depending on whether soluble factors in the ECM can bind the cell surface receptors, which link membrane HSPGs as co-receptor. In 143B over-expressing glypican-5 cells, GPC5 and the PG pattern associated could be implicated in the rearrangement of cytoskeleton, especially in response to Collagen type III, where the cells show a formation of protrusion lamellipodia-like, Fibronectin and Laminin-1, whereas seems not to interfere with the formation of stress fibers when adhere on the other ECM molecules analyzed; besides, GPC5 seems to have a preference of adhesion to Laminin-1 and Vitronectin. In contrast, when it was observed the invasive ability of $GPC5^{+}$ cells on the same molecules of ECM, cells over-expressing glypican-5 migrate more pronouncedly on Vitronectin and Collagen type III and less on Fibronectin. These results suggest that,

when abundantly expressed on the cell surface, GPC5 differentially regulate cell adhesion and migration to selected ECM components and future perspective are to investigate the significance of this phenomenon. GPC5 seems to influence also the overall locomotory abilities of the cells. Other ways which syndecans and glypicans can relay our contribute to signal transduction are their shedding in the extracellular environment or their internalization within the endocytotic vesicle, in which the GPI-anchored protein can be endocytosed and degraded, cleaved and released, vesiculated, or exchanged onto another cell[102]. In the preliminary analysis of post translational modification of glypican-5, seems that GPC5 is not released in the extracellular environment, and probably it is favourite an internalization process; remain to define more in particular this outcome, because this results could be due to a partial or total lack of enzyme for the cleavage on the GPI anchor or there are post-translational modification that alter the amino acidic structure of the protein and prevent proteases/lipases from doing cleavage or more simply the experimental procedure don't allows us to detect the N-terminal of the glypican-5 in the medium.

Gaining better inside into the mechanism that result in the behavior observed of these models, could be a new perspective in the understanding how these molecules regulate different aspects of cancer biology. "*Glycoprofiling*" cancer cells by mapping cell surface HSPGs might provide an opportunity to develop important prognostic tool for cancer detection and staging. The cell surface PG pattern could supply to all the scientific community important data to the prediction of cancer growth or metastatic potential, as well as its potential response to therapy. It is reasonable to think that a more deep knowledge of glypicans involvement in normal and pathological processes, as well as the identification of correlate signaling, should provide a wider clinical approach for the development of targeted therapeutics.

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6. BIBLIOGRAPHY

[1] Bishop, J.R., Schuksz, M., Esko, J.D. (2007) Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature, 446, 1030-1037

[2] Wegrowski, Y., Maquart F.X. (2004) Involvement of stromal proteoglycans in tumour progression. Critical Reviews in Oncology/Hematology, 49, 259-268

[3] Hardingham, TE., Fosang, AJ. (1992) Proteoglycans: many forms and many functions. FASEB J , 6, 861–70

[4] Beaty, NB., Mello, RJ. (1987) Extracellular mammalian polysaccharides: glycosaminoglycans and proteoglycans. J Chromatogr , 418, 187–222

[5] Iozzo, RV. (1999) The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. J Biol Chem, 274, 18843–6

[6] Iozzo, RV. (1998) Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem, 67, 609–52

[7] Iozzo, RV., Murdoch, AD. (1996) Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J, 10, 598–614

[8] Sames, K. (1994) Glycosaminoglycans, proteoglycans and aging. EXS, 70, 243–74

[9] Timar, J., Lapis, K., Dudas, J., et al. (2002) Proteoglycans and tumor progression: Janus-faced molecules with contradictory functions in cancer. Semin Cancer Biol, 12, 173–86

[10] Functional and Molecular Glycobiology 2002. Brooks SA, Dwek, MV, Schumacher, U. Bios Scientific Publishers.

[11] Molecular Biology of the Cell (3rd Edition). Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Garland Publishing

[12] Bernfield, M., Götte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., Zako, M. (1999) Function of cell surface. Annu. Rev. Biochem., 68, 729–777

[13] Kato, M.,Wang, H., Kainulainen, V., Fitzgerald, ML., Ledbetter, S., et al. (1998) Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. Nat. Med., 4, 691–97

[14] Lopez-Casillas, F., Payne, HM., Andres, JL., Massagu´e, J. (1994) Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding

and GAG attachment sites. J. Cell Biol., 124, 557–68

[15] Fitzgerald, ML., Wang, Z., Park, PW., Murphy, G., Bernfield, M. (2000) Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase. J. Cell Biol., 148, 811–24

[16] Park, PW., Pier, GB., Hinkes, MT., Bernfield, M. (2001) Exploitation of syndecan-1 shedding by Pseudomonas aeruginosa enhances virulence. Nature, 411, 98–102

[17] Cano-Gauci, D.F., et al. (1999) Glypican-3 deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. J. Cell Biol., 146, 255-264

[18] Chiao, E., et al (2002) Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndromeis independent of IGF signaling. Dev. Biol., 243, 185-206

[19] Hsueh, Y.P., Yang, F.C., Kharazia, V., Naisbitt, S., Cohen, A.R., Weinberg, R.J., Sheng, M. (1998) Direct interaction of CASK/LIN-2 and syndecan heparan sulfate proteoglycan and their overlapping distribution in neuronal synapses. J.Cell.Biol., 142, 139-151

[20] Leppa, S., Mali, M., Miettinen, H.M., Jalkanen, M. (1992) Syndecan expression regulates cell morphology and growth of mouse mammary epithelial tumor cells. PNAS, 89, 932-936

[21] Kato, M., Saunders. S., Nguyen. H., Bernfield. M. (1995) Loss of cell surface Syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like. Mol. Biol. Cell. 6, 559-576

[22] Wiksten, J.P., Lundin, J., Nordling, S., Lundin, M., Kokkola, A., von Boguslawski, K., Haglund, C. **(**2001) Epithelial and stromal syndecan-1 expression as predictor of outcome in patients with gastric cancer. Int J Cancer., 95(1), 1-6

[23] Barbareschi, M., Maisonneuve, P., Aldovini, D., Cangi, M.G., Pecciarini, L., Angelo Mauri, F., Veronese, S., Caffo, O., Lucenti, A., Palma, P.D., Galligioni, E., Doglioni, C. (2003) High syndecan-1 expression in breast carcinoma is related to an aggressive phenotype and to poorer prognosis. Cancer., 98(3), 474-83.

[24] Klass, C.M., Couchman, J.R., Woods, A. (2000) Control of extracellular matrix assembly by syndecan-2 proteoglycan. J.Cell. Biol., 113, 493-506

[25] Beauvais DL.M., Rapraeger, A.C. (2004) Syndecans in tumor cell adhesion and signaling. Reprod Biol Endocrinol.2, 3. Review

[26] Tkachenko, E., Rhodes, J.M., Simons, M. (2005) Syndecans: new kids on the signaling block. Circ Res., 96(5), 488-500. Review.

[27] Park, H., Kim, Y., Lim, Y., Han, I., Oh, ES. (2002) Syndecan-2 mediates adhesion and proliferation of colon carcinoma cells. J Biol Chem.,277(33), 29730-6

[28] Kinnunen, A., Niemi, M., Kinnunen, T., Kaksonen, M., Nolo, R., Rauvala, H. (1999) Heparan sulphate and HB-GAM (heparin-binding growth-associated molecule) in the development of the thalamocortical pathway of rat brain. Eur J Neurosci., 11(2), 491-502.

[29] Simons, K., Vaz, W.L. (2004) Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struct., 33, 69-95. Review

[30] Midwood, K.S., Williams, L.V., Schwarzbauer, J.E. (2004) Tissue repair and the dynamics of the extracellular matrix. Int J Biochem Cell Biol., 36(6), 1031-7. Review

[31] Gulyás, M., Hjerpe, A. (2003) Proteoglycans and WT1 as markers for distinguishing adenocarcinoma, epithelioid mesothelioma, and benign mesothelium.
J Pathol. , 199(4), 479-87

[32] Yung, S., Woods, A., Chan, T.M., Davies, M., Williams, J.D., Couchman, J.R. (2001) Syndecan-4 up-regulation in proliferative renal disease is related to microfilament organization. FASEB J., 15(9), 1631-3.

[33] Lopes, C.C., Dietrich, C.P., Nader, H.B. (2006) Specific structural features of syndecans and heparan sulfate chains are needed for cell signaling. Braz J Med Biol Res., 39(2), 157-67, Review

[34] Filmus, J., Selleck, S.B. (2001) Glypicans: proteoglycans with a surprise. J. Clin. Invest., 108, 497– 501

[35] Filmus, J., Song, H.H. (2000) Proteoglycans, in: R.V. Iozzo (Ed.), Marcel Dekker, New York, 161– 176

[36] Watanabe, K., Yamada, H., Yamaguchi, Y. (1995) K-Glypican: A Novel GPI-anchored Heparan Sulfate Proteoglycan That Is Highly Expressed in Developing Brain and Kidney. J. Cell Biol., 130, 1207– 1218

[37] Veugelers, M., De Cat, B., Ceulemans, H., Bruystens, A.M., Coomans, C., Durr, J., Vermeesch, J., Marynen, P., David, G. (1999) Glypican-6, a New Member of the Glypican Family of Cell Surface Heparan Sulfate Proteoglycans. J. Biol. Chem., 274, 26968– 26977.

[38] Saunders, S., Paine-Saunders, S., Lander, A.D. (1997) Expression of the Cell Surface Proteoglycan Glypican-5 Is Developmentally Regulated in Kidney, Limb, and Brain. Dev. Biol., 190, 78–93

[39] Stipp, C.S., Litwac, E.D., Lander, A.D. (1994) Cerebroglycan: an integral membrane heparan sulfate proteoglycan that is unique to the developing nervous system and expressed specifically during neuronal differentiation. J. Cell Biol., 124, 149– 160.

[40] David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J., Van Den Berghe, H. (1990) Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. J. Cell Biol., 111, 3165– 3176.

[41] De Cat, B., David, G., (2001) Developmental roles of the glypicans. Semin Cell Dev. Biol., 12, 117-125

[42] Filmus, J. (2001) Glypicans in growth control and cancer. Glycobiology, 11(3), 19R-23R

[43]Song, H.H., Filmus, J. (2002) The role of glypicans in mammalian development. Biochim Biophys Acta., 1573(3), 241-246. Review

[44] Filmus, J., Shi, W., Wong, Z.M., Wong, M.J. (1995) Identification of a new membrane-bound heparan sulphate proteoglycan. Biochem J.,311, 561-565

[45] Liang, Y., Häring, M., Roughley, P.J., Margolis, R.K., Margolis, R.U. (1997) Glypican and biglycan in the nuclei of neurons and glioma cells: presence of functional nuclear localization signals and dynamic changes in glypican during the cell cycle. J Cell Biol., 139(4), 851-864.

[46] Veugelers, M., Vermeesch, J., Reekmans, G., Steinfield, R., Marynen, P., David, G. (1997) Characterization of Glypican-5 and Chromosomal Localization of Human GPC5, a New Member of the Glypican Gene Family. Genomics, 40, 24-30

[47] Capurro, M., Shi, W., Sandal, S., Filmus, J. (2005) Processing by Convertase Is Not Required for Glypican-3-induced Stimulation of Hepatocellular Carcinoma Growth. J. Biol. Chem, 280(50), 41201-41206

[48] Brown, D.A., Rose, J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell, 68, 533-544

[49] Simons, K., Ikonen, E. (1997) Functional rafts in cell membranes. Nature, 387, 569-572

[50] Fransson, L.A., Edgren, G., Havsmark, B., Schmidtchen, A. (1995) Recycling of a glycosylphosphatidylinositol-anchored heparan sulphate proteoglycan (glypican) in skin fibroblasts. Glycobiology, 5(4), 407-15.

[51] Häcker, U., Nybakken, K., Perrimon, N. (2005) Heparan sulphate proteoglycans: the sweet side of development. Nat Rev Mol Cell Biol., 6(7), 530-41. Review

[52] Lin, X. (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. Development., 131(24), 6009-21. Review

[53] Filmus, J., Capurro, M., Rast, J. (2008) Glypicans. Genome Biol., 9(5), 224

[54] Giraldez, A.J., Copley, R.R. and Cohen, S.M. (2002) HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. Dev. Cell, 2, 667–676

[55] Gerlitz, O. and Basler, K. (2002) Wingful, an extracellular feedback inhibitor of Wingless. Genes Dev., 16, 1055–1059

[56] Traister, A., Shi, W., Filmus J. (2008) Mammalian Notum induces the release of glypicans and other GPI-anchored proteins from the cel surface. Biochem. J., 410, 503-511

[57] Grozdanov, N.P., Yovchev, M.I. and Dabeva, M.D. (2006) The oncofetal protein glypican-3 is a novel marker of hepatic progenitor/oval cells. Laboratory Investigation, 86, 1272–1284

[58] Li, M., Choo, B., Wong, Z.M., Filmus, J., Buick, R.N. (1997) Expression fo OCI-5/Glypican-3 during intestinal morphogenesis: Regulation by cell shape in intestinal epithelial cells. Exp. Cell Res., 235, 3-12

[59] Pilia, G., Hughes-Benzie, R.M., MacKenzie, A., Baybayan, P., Chen, E.Y., Huber, R., Neri, G., Cao, A., Forabosco, A., Schlessinger, D. (1996) Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat. Genet., 12, 241-247

[60] Pellegrini, M., Pilia, G., Pantano, S., Lucchini, F., Uda, M., Fumi, M., Cao, A., Schlesinger, D., Forabosco, A. (1998) Gpc3 expression correlates with the phenotype of the Simpson-Golabi-Behmel syndrome. Dev.Dyn., 213, 431-439

[61] Lin, H., Huber, R., Schlessinger, D., et al. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. (1999) Cancer Res, 59, 807–810

[62] Murthy SS, Shen T, De Rienzo A, et al. (2000) Expression of GPC3, an X-linked recessive overgrowth gene, is silenced in malignant mesothelioma. Oncogene, 19, 410–416

[63] Xiang, Y.Y., Ladeda, V., Filmus, J. (2001) Glypican -3 expression is silenced in human breast cancer. Oncogene, 20, 7408–7412

[64] Hsu, H.C., Cheng, W., Lai, P.L. (1997) Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. Cancer Res, 57, 5179–5184

[65] Lage, H., Dietel, M., Froschle, G., et al. (1998) Expression of the novel mitoxantrone resistance associated gene MXR7 in colorectal malignancies. Int J Clin Pharmacol Ther, 36, 58–60

[66] Saikali, Z., Sinnett, D. (2000) Expression of glypican3 (GPC3) in embryonal tumors. Int J Cancer, 89, 418–422

[67] Zhu, Z.W., Friess, H., Wang, L., et al. (2001) Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut, 48, 558–564

[68] Yamauchi, N., Watanabe, A., Hishinuma, M., Ohashi, K., Midorikawa, Y., Morishita, Y., Niki, T., Shibahara, J., Mori, M., Makuuchi, M., Hippo, Y., Kodama, T., Iwanari, H., Aburatani, H., Fukayama, M. (2005) The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. Mod Pathol.,18, 1591-8

[69] Capurro, M., Wanless, I.R., Sherman, M., et al. (2003) Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology, 125, 81–90

[70] Capurro, M.I., Xiang, Y.Y., Lobe, C., Filmus, J. (2005) Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res., 15, 6245-54

[71] Nakatsura, T., Yoshitake, Y., Senju, S., et al. (2003) Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun, 306, 16–25

[72] Hippo Y, Watanabe K, Watanabe A, et al. (2004) Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res, 64, 2418–23

[73] Litwack, E.D., Ivins, J.K., Kumbasar, A., Paine-Saunders, S., Stipp, C.S., Lander, A.D. (1998) Expression of the heparan sulfate proteoglycan glypican-1 in the developing rodent. Dev.Dyn., 211, 72-87

[74] Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., Perrimon, N. (2001) Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development, 126, 87-94

[75] Sangai, T., Ishii, G., Kodama, K, et al. (2005) Effect of differences in cancer cells and tumor growth sites on recruiting bone marrow-derived endothelial cells and myofibroblasts in cancer-induced stroma. Int J Cancer. , 115, 885–892

[76] Kleeff, J., Ishiwata, T., Kumbasar, A, et al. (1998) The cell-surface heparan sulfate proteoglycan glypican-1 is an important regulator of growth factor action in pancreatic carcinoma cells, and is overexpressed in human pancreatic cancer. J Clin Invest., 102, 1662–1673

[77] Korc, M. (2007) Pancreatic cancer-associated stroma production. Am J Surg., 194, S84-6. Review

[78] Kayed, H., Kleeff, J., Keleg, S., Jiang, X., Penzel, R., Giese, T., Zentgraf, H., Büchler, M.W., Korc, M., Friess, H. (2006) Correlation of glypican-1 expression with TGF-beta, BMP, and activin receptors in pancreatic ductal adenocarcinoma. Int J Oncol., 29, 1139-48

[79] Ivins, J.K., Litwack, E.D., Kumbasar, A., Stipp, C.S., Lander, A.D. (1997) Cerebroglycan, a developmentally regulated cell-surface heparan sulfate proteoglycan, is expressed on developing axons and growth cones. Dev.Biol., 184, 320-332

[80] Hagihara, H., Watanabe, K., Chun, J., Yamaguchi, Y. (2000) Glypican-4 is an FGF2-binding heparan sulfate proteoglycan expressed in neural precursor cells. Dev.Dyn., 219, 353-367

[81] Karihaloo, A., Kale, S., Rosenblum, N. D. and Cantley, L. G. (2004) Hepatocyte growth factor-mediated renal epithelial branching morphogenesis is regulated by glypican-4 expression. Mol. Cell. Biol., 24, 8745–8752

[82] Fico, A., Maina, F., Dono, R. (2007) Fine-tuning of cell signaling by glypicans. Cell. Mol. Life Sci., [Epub ahead of print]

[83] Saunders, S., Paine Saunders, S., Lander, A.D. (1996) Glypican-5: Molecular cloning of a novel member of the glypican gene family. Mol. Biol. Cell, 7, 237a

[84] Litwack ED, Stipp CS, Kumbasar A, Lander AD. **(**1994**)** Neuronal expression of glypican, a cell-surface glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan, in the adult rat nervous system. J Neurosci., 14(6), 3713-24

[85] Brown S, Gersen S, Anyane-Yeboa K, Warburton D. (1993) Preliminary definition of a "critical region" of chromosome 13 in q32: report of 14 cases with 13q deletions and review of the literature. Am J Med Genet., 45(1), 52-9. Review

[86] Brown S, Russo J, Chitayat D, Warburton D. (1995) The 13q- syndrome: the molecular definition of a critical deletion region in band 13q32. Am J Hum Genet., 57(4), 859-66

[87] Yu W, Inoue J, Imoto I, Matsuo Y, Karpas A, Inazawa J. (2003) GPC5 is a possible target for the 13q31-q32 amplification detected in lymphoma cell lines. J Hum Genet., 48(6), 331-5

[88] de Leeuw RJ, Davies JJ, Rosenwald A, Bebb G, Gascoyne RD, Dyer MJ, Staudt LM, Martinez-Climent JA, Lam WL. (2004) Comprehensive whole genome array CGH profiling of mantle cell lymphoma model genomes. Hum Mol Genet., 13(17), 1827-37

[89] Schraders M, Pfundt R, Straatman HM, Janssen IM, van Kessel AG, Schoenmakers EF, van Krieken JH, Groenen PJ. (2005) Novel chromosomal imbalances in mantle cell

lymphoma detected by genome-wide array-based comparative genomic hybridization. Blood., 105(4), 1686-93.

[90] Williamson D, Selfe J, Gordon T, Lu YJ, Pritchard-Jones K, Murai K, Jones P, Workman P, Shipley J. (2007) Role for amplification and expression of glypican-5 in rhabdomyosarcoma. Cancer Res., 67(1), 57-65

[91] Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U. (2002) Roles of heparan-sulphate glycosaminoglycans in cancer. Nat Rev Cancer., 2(7), 521-8. Review

[92] Liu D, Shriver Z, Qi Y, Venkataraman G, Sasisekharan R. (2002) Dynamic regulation of tumor growth and metastasis by heparan sulfate glycosaminoglycans. Semin Thromb Hemost.,28(1), 67-78. Review

[93] Sasisekharan R, Venkataraman G. (2000) Heparin and heparan sulfate: biosynthesis, structure and function. Curr Opin Chem Biol., 4(6), 626-31

[94] Fuki IV, Meyer ME, Williams KJ. (2000)Transmembrane and cytoplasmic domains of syndecan mediate a multi-step endocytic pathway involving detergent-insoluble membrane rafts. Biochem J., 351(3), 607-12

[95] Belenkaya TY, Han C, Yan D, Opoka RJ, Khodoun M, Liu H, Lin X. (2004) Drosophila Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans. Cell, 119(2), 231-44

[96] Marois E, Mahmoud A, Eaton S. (2006)The endocytic pathway and formation of the Wingless morphogen gradient. Development, 133(2), 307-17

[97] Cattaruzza S, Perris R. (2006) Approaching the proteoglycome: molecular interactions of proteoglycans and their functional output. Macromol Biosci. , 6(8), 667-80. Review

[98] Schlessinger J, Lax I, Lemmon M. (1995) Regulation of growth factor activation by proteoglycans: what is the role of the affinity receptors? Cell, 83(3), 357-60. Review

[99] Taylor, K.R. (2006) Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. et al. FASEB J.

[100] Blackhall FH, Merry CL, Davies EJ, Jayson GC. (2001) Heparan sulfate proteoglycans and cancer. Br J Cancer, 85(8), 1094-8. Review

[101] Grisaru S, Cano-Gauci D, Tee J, Filmus J, Rosenblum ND. (2001) Glypican-3 modulates BMP- and FGF-mediated effects during renal branching morphogenesis. Dev Biol.,231(1), 31-46

[102] Censullo, P., Davitz, M.A. (1994) How GPI-anchored proteins turnover: or where do they go after arrival at the plasma membrane. Semin Immunol. , 6(2), 81-8. Review

[103] Freeman, M., Gurdon, J.B. (2002) Regulatory principles of developmental signaling. Annu. Rev. Cell. Dev. Biol., 18, 515-539

[104] Sharom, F.J., Radeva, G. (2004) GPI-anchored protein cleavage in the regulation of transmembrane signals. Subcell Biochem., 37, 285-315. Review

[105] Tsujioka, H., Misumi, Y., Takami, N., Ikehara, Y. (1998) Posttranslational modification of glycosylphosphatidylinositol (GPI)-specific phospholipase D and its activity in cleavage of GPI anchors. Biochem Biophys Res Commun., 251(3), 737-43

[106] Poon, G.M., Gariépy, J. (2007) Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. Biochem Soc Trans., 35(Pt 4), 788-93. Review

[107] Chatterjee, S., Smith, E.R., Hanada, K., Stevens, V.L., Mayor, S. (2001) GPI anchoring leads to sphingolipid-dependent retention of endocytosed proteins in the recycling endosomal compartment. EMBO J., 20(7), 1583-92

[108] Simons, K., Toomre, D. (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol., 1(1), 31-9. Review. Erratum in: Nat Rev Mol Cell Biol 2001 Mar;2(3):216

[109] Nguyen, D.X., Massagué, J. Genetic determinants of cancer metastasis. Nature Reviews Genetics 8, 341-352 (May 2007)

[110] Statistics adapted from the American Cancer Society's publication, Cancer Facts and Figures 2008; Cancer.Net Editorial Board, 08/08

[111] Statistics adapted from the American Cancer Society's publication, Cancer Facts & Figures 2008, and from data from the National Cancer Institute and Memorial Sloan-Kettering Cancer Center

[112] Gustafson, P. (1994) Soft tissue sarcoma. Epidemiology and prognosis in 508 patients. Acta Othop. Scand. Suppl, 259, 1-31

[113] Parker, S.L., Tong, T., Bolden, S., Wingo, P.A. (1996) Cancer statistics, 1996. Cancer J. Clin., 46, 5-27

[114] Kotilingam, D., Lev, D.C., Lazar, A.J., Pollock, R.E. (2006) Staging soft tissue sarcoma: evolution and change. CA Cancer J. Clin., 56(5), 282-91; quiz 314-5. Review

[115] Lahat, G., Tuvin, D., Wei, C., Anaya, D.A., Bekele, B.N., Lazar, A.J., Pisters, P.W., Lev, D., Pollock, R.E. (2008) New perspectives for staging and prognosis in soft tissue sarcoma. Ann. Surg. Oncol., 15(10), 2739-48. Epub 2008 Jun 3

[116] Fjeldstad, K., Kolset, S.O. (2005) Decreasing the metastatic potential in cancers--targeting the heparan sulfate proteoglycans. Curr. Drug Targets, 6(6), 665-82

[117] Baumhoer, D., Tornillo, L., Stadlmann, S., Roncalli, M., Diamantis, E.K., Terracciano, L.M. (2008) Glypican 3 expression in human nonneoplastic, preneoplastic, and neoplastic tissues: a tissue microarray analysis of 4,387 tissue samples. Am J Clin Pathol. , 129(6), 899-906

[118] Baer, C., Nees, M., Breit, S., Selle, B., Kulozik, A.E., Schaefer, K.L., Braun, Y., Wai, D., Poremba, C. (2004) Profiling and functional annotation of mRNA gene expression in pediatric rhabdomyosarcoma and Ewing's sarcoma. Int J Cancer, 110(5), 687-94

[119] Weber-Hall, S., Anderson, J., McManus, A., Abe, S., Nojima, T., Pinkerton, R., Pritchard-Jones, K., Shipley, J. (1996) Gains, losses, and amplification of genomic material in rhabdomyosarcoma analyzed by comparative genomic hybridization. Cancer Res., 56(14), 3220-4

[120] Gordon, A.T., Brinkschmidt, C., Anderson, J., Coleman, N., Dockhorn-Dworniczak, B., Pritchard-Jones, K., Shipley J. (2000) A novel and consistent amplicon at 13q31 associated with alveolar rhabdomyosarcoma. Genes Chromosomes Cancer., 28(2), 220-6

[121] Chu, C.L., Buczek-Thomas, J.A., Nugent, M.A. (2004) Heparan sulphate proteoglycans modulate fibroblast growth factor-2 binding through a lipid raft-mediated mechanism. Biochem J., 379(Pt 2), 331-41. Erratum in: Biochem J., 380(Pt 3), 951

[122] Knuutila, S., Autio, K., Aalto, Y. (2000) Online access to CGH data of DNA sequence copy number changes. Am J Pathol., 157(2), 689. Erratum in: Am J Pathol., 157(4), 1413

[123] Schmidt, H., Bartel, F., Kappler, M., Würl, P., Lange, H., Bache, M., Holzhausen, H.J., Taubert, H. (2005) Gains of 13q are correlated with a poor prognosis in liposarcoma. Mod Pathol., 18(5), 638-44

[124] WHO, World Health Organization. Data and statistics, 2009

[125] Medscape's Head and Neck Cancer Resource Center

[126] Cotran, Kumar, & Collins: Pathologic Basis of Disease, 6th Edition.

[127] Neville, Damm, Allen & Boquot: Oral and Maxillofacial Pathology, 2nd Edition

[128] Sanderson, R.D., Lalor, P., Bernfield, M. (1989) B lymphocytes express and lose syndecan at specific stages of differentiation. Cell Regul., 1, 27–35

[129] Stanley, M.J., Liebersbach, B.F., Liu, W., Anhalt, D.J., Sanderson, R.D. (1995) Heparan sulfate-mediated cell aggregation. Syndecans-1 and -4 mediate intercellular adhesion following their transfection into human B lymphoid cells. J Biol Chem, 270, 5077–83.

[130] Hayashi, K., Hayashi, M., Jalkanen, M., Firestone, J.H., Trelstad, R.L., Bernfield, M. (1987) Immunocytochemistry of cell surface heparan sulfate proteoglycan in mouse tissues: a light and electron microscopic study. J Histochem Cytochem, 35, 1079–88.

[131] Soukka, T., Pohjola, J., Inki, P., Happonen, R.P. (2000) Reduction of syndecan-1 expression is associated with dysplastic oral epithelium. J Oral Pathol Med., 29, 308-13

[132] Inki, P., Larjava, H., Haapasalmi, K., Miettinen, H.M., Grenman, R., Jalkanen, M. (1994) Expression of syndecan-1 is induced by differentiation and suppressed by malignant transformation of human keratinocytes. Eur J Cell Biol., 63, 43-51.

[133] Kurokawa, H., Matsumoto, S., Murata, T., Yamashita, Y., Tomoyose, T., Zhang, M., Fukuyama, H., Takahashi, T. 2003Immunohistochemical study of syndecan-1 down-regulation and the expression of p53 protein or Ki-67 antigen in oral leukoplakia with or without epithelial dysplasia. J Oral Pathol Med, 32, 513-21

[134] Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72:248-254.

[135] Zor, T. and Selinger, Z (1995) Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal. Biochem. 236:302-8

[136] http://www.pathology.wisc.edu/labs/rapraeger/

[137] Perrimon, N., Bernfield, M. (2000) Specificities of heparan sulphate proteoglycans in developmental processes. Nature, 404, 725-728

[138] Conrad, H.E. (1998) Heparin-Binding Proteins. Academic Press, San Diego / 92

[139] Liù, D., Shriver, Z., Venkataraman, G., El Shabrawi, Y. & Sasisekharan, R. (2002) Tumor cell surface heparan sulphate as cryptic promoters or inhibitors of tumor growth and metastasis. Proc. Nat. Acad. Sci. USA, 99, 568-573