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GANGLIOSIDE-DEPENDENT MEMBRANE ORGANIZATION CONTROLLING THE ADHESION AND MOTILITY OF HUMAN OVARIAN CANCER CELLS

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ABSTRACT

Glycosphingolipids, due to their tendency to form laterally separated liquid-ordered phases, possess a high potential for the creation of order in biological membranes. The formation of glycosphingolipid-rich membrane domains within the membrane has profound consequences on the membrane organization at different levels, and on the conformational and biological properties of membrane-associated proteins and multimolecular protein complexes (1).

Glycosphingolipids modulate several signal transduction processes controlling cell proliferation, survival, differentiation, and transformation. Since alterations in the expression of carbohydrate epitopes associated with glycosphingolipids are frequent in tumors, it has been hypothesized that glycosphingolipids could play important roles in modulating some of the properties of tumor cells. In fact, some tumors are characterized by the ability to manipulate sialylation processes determining the formation of antigenic determinants resulting from an "aberrant glycosylation" and affecting cell homeostasis, altering the normal signaling pathways (2). Thus, an ever-increasing interest to this regard is being devoted to gangliosides, sialic acid-containing glycolipids, and to the enzymes affecting sialylation. Both sialyltransferases and sialidases seem to be involved in the phenomenon of aberrant sialylation in tumor cells.

The genetic (stable overexpression of sialyltransferase I - SAT-I or GM3 synthase) or pharmacological (selective pressure by *N*-(4-hydroxyphenyl)retinamide) manipulation of A2780 human ovarian carcinoma cells allowed us to obtain monoclonal cells characterized by higher GM3 synthase activity respect to wild type cells (3-5). High GM3 synthase expression resulted in 1) elevated ganglioside levels, 2) reduced *in vitro* cell motility and increased adhesion to fibronectin, 3) enhanced expression of the membrane adaptor protein caveolin-1, an integral membrane protein playing multiple roles as negative regulator in the progression of several types of human tumors (6,7). The correlation between high ganglioside levels and decreased motility/increased adhesion were confirmed through administration of exogenous gangliosides which was not only able to strongly reduce *in vitro* cell motility, but also to significantly increase cell adhesive ability to fibronectin in wild type cells, low GM3 synthase expressing A2780 cells. Furthermore, in high GM3 synthase expressing clones, such as A2780/SAT-I cells, ganglioside depletion by treatment with the glucosylceramide synthase inhibitor D-PDMP was able to strongly reduce adhesion and to increase cell motility.

The α5β1 integrin, which mediates cell adhesion, is the most expressed integrin heterodimers in A2780 cells. Since fibronectin is the preferential ligand of integrin α5β1, it has been hypothesized that this integrin heterodimer might be involved in the regulation of adhesion and motility in human ovarian carcinoma cells. On the other hand, A2780/SAT-I cells also showed an increased adhesion to laminin and vitronectin suggesting a possible role of their respective integrin receptors in the regulation of GM3-mediated adhesion.

The role of a glycosphingolipid/caveolin-1 signaling complex in the negative regulation of A2780 cells motility has been reported, showing that high levels of caveolin-1 and high levels of gangliosides are necessary, but not sufficient, to down-regulate tumor cell motility (5). In GM3 synthase expressing cells, caveolin-1 and gangliosides were highly enriched in detergent-resistant membrane fractions (DRM) prepared in the presence of Triton X-100. D-PDMP treatment determined changes in the lipid distribution of several lipids in sucrose gradient fractions, and also altered protein distribution determining a shift of both caveolin-1 and c-Src, also involved in the previously mentioned complex signaling pathway, from the DRM fraction to intermediate fraction. Integrins, particularly α5 and β1 integrin subunits, following ganglioside depletion move from the high density fraction to DRM and intermediate fractions. D-PDMP treatment also affected protein association with caveolin-1, determining an increased association of this protein, a potential molecular organizer, with integrin subunits α5 and β1 without affecting the total level of proteins.

The *in vitro* adhesion of A2780/SAT-I cells was markedly higher in caveolin-1 silenced cells compared with scramble sequence transfected cells, suggesting a leading role of caveolin-1 in the regulation of the cell adhesion signal in this cell model. On the other hand, treatment of A2780 cells with exogenous gangliosides only slightly increased the expression of caveolin-1; while it markedly increased the phosphorylation of caveolin-1 at tyrosine 14. Conversely, ganglioside depletion in high GM3 synthase-expressing clones by D-PDMP treatment markedly reduced caveolin-1 phosphorylation. These data suggest that phosphorylation of caveolin-1, rather than caveolin-1 total level, is controlled by gangliosides and is crucial in the control of tumor cell adhesion.

These data suggest a novel role of gangliosides in regulating tumor cell adhesion and motility, by affecting the organization of a signaling complex organized by caveolin-1, and imply that GM3 synthase is a key target for the regulation of cell motility and adhesion in human ovarian carcinoma.

INTRODUCTION

Glycosphingolipids

Overview

All eukaryotic cells are surrounded by a membrane composed of a lipid bilayer, whose chemical nature and essential role in cell permeability were first proposed around a hundred years ago. Today it is known that there are three major classes of lipids in eukaryotic cell membranes, namely glycerolipids, sphingolipids, and sterols, whose biochemical and biophysical properties vary considerably and impact upon their function (8). Glycerophospholipids are by far the main lipids of eukaryotic cell membranes. Sphingolipids are minor cell components, mainly residing in the external layer of the plasma membrane (9) with the hydrophilic headgroup protruding toward the extracellular environment. They constitute a family of amphipathic lipids found in essentially all animals, plants and fungi, some prokaryotic organisms and viruses, and involved in numerous and important biological functions (10). Ceramide is the simplest sphingolipid: it is formed by a long chain sphingoid base linked to a fatty acid through an amide bound. In mammalian tissues the most common sphingoid base is sphingosine (2S,3R-d-erythro-2-amino-1,3-octadec-4E-ene-diol, also called trans-4-sphingenine), a 18 carbon atoms primary amine with a double bond in position 4,5 and two hydroxyl groups in position 1 and 3. Homologous lipids with a different length of the carbon chain or with a saturated chain, sphinganine or 4-hydroxy-sphinganine, are present in cells in minor amount (11). Ceramide is the backbone of all complex sphingolipids which are characterized by the presence of a charged group linked to the hydroxylated group in position 1 of the sphingoid base (12). The polar group, that defines the specific sphingolipid class, is a phosphate group in ceramide-1-phosphate, phosphorylcholine in sphingomyelin, monosaccharides in cerebrosides, one or more sugar residues linked with a β-glycosidic bond in complex glycosphingolipids. Glycosphingolipids are the most structurally diverse class of complex sphingolipids, and are normally classified as acidic, neutral or basic (8,13). They are ubiquitous components of mammal cell membranes, but are particularly abundant in the nervous system.

A particular class of acidic glycosphingolipids, named gangliosides, was discovered in 1936 by Ernest Klenk in the central nervous system. Gangliosides are sialic acid-containing glycosphingolipids, and have been considered to be involved in the development, differentiation, and function of nervous systems in vertebrates (14). In

cells, gangliosides are primarily localized in the extracellular leaflet of the plasma membrane where they are not homogenously distributed (15). On the cell surface, gangliosides are involved in cell-cell recognition, adhesion and signal transduction within specific cell surface microdomains, named caveolae (16), lipid rafts (17), or glycosphingolipid-enriched microdomains (18) with other membrane components such as sphingomyelin and cholesterol. Evidence is accumulating that gangliosides are colocalized in the microdomain structures with signaling molecules and adhesion molecules which suggests their involvement in the modulation of process such as cell proliferation, survival, adhesion, and neuronal differentiation. In addition to cell plasma membranes, gangliosides have been shown to be present on nuclear membranes, and they have recently been proposed to play important roles in modulating intracellular and intranuclear calcium homeostasis and the ensuing cellular functions (19). Many different experimental approaches, leading eventually to alterations in the organization of the plasma membrane due to quali- or quantitative changes in glycosphingolipid content or pattern, have been proven to be very effective in modulating the above mentioned cell functions. On the other hand, catabolic fragments derived from plasma membrane sphingolipids (ceramide, sphingosine, and sphingosine-1-phosphate) ermerged as a class of lipid mediators capable of modulating cell proliferation, differentiation, motility or apoptotic cell death.

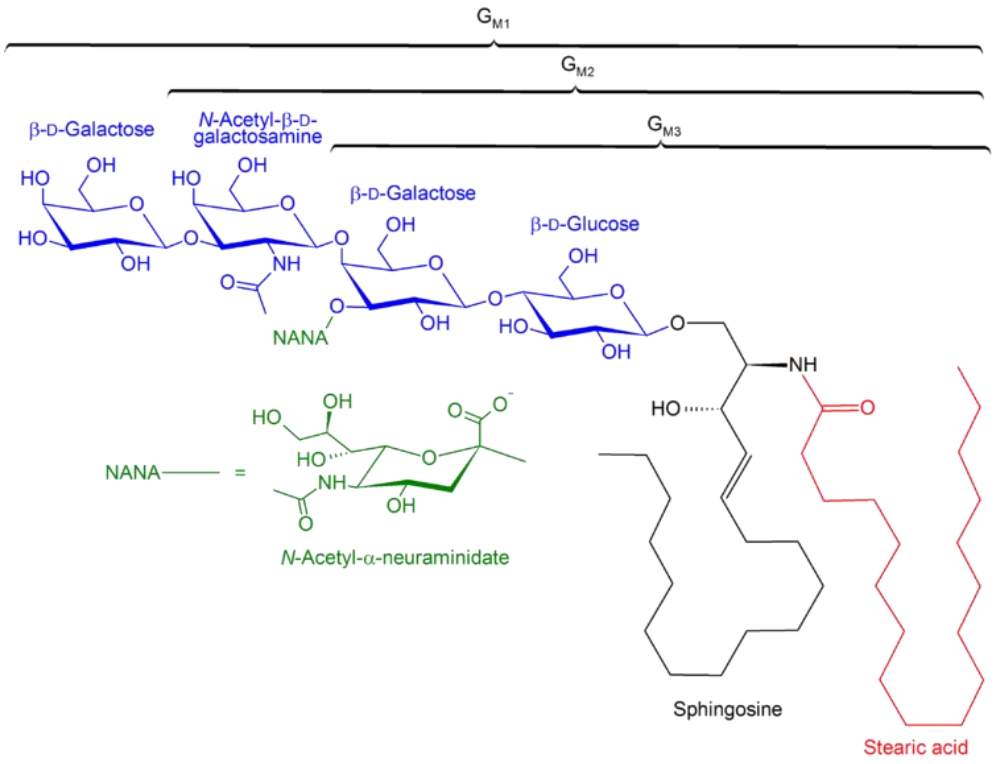


Figure 1. Structure of GM1, GM2 and GM3 ganglioside

Glycosphingolipids biosynthesis, trafficking and degradation

The biochemical pathways of glycosphingolipids metabolism and the intracellular sites of synthesis and degradation, in the endoplasmic reticulum/Gogli apparatus and lysosomes, respectively, have been characterized extensively over the past couple of decades (20,21). The biosynthesis of glycosphingolipids starts at the cytosolic leaflet of membranes of the endoplasmic reticulum (ER) where ceramide is synthesized by a sequence of three enzyme-catalysed reactions from L-Serine and two molecules of coenzyme A (CoA) activated fatty acid, namely palmitoyl-CoA (22). After its synthesis, ceramide equilibrates to the luminal side of the ER where it can be transformed into galactosylceramide (GalCer) by ceramide galactosyltransferase (23). GalCer after its synthesis traffics through the Golgi apparatus where it may be sulfated (24) or glycosylated (22). Ceramide is also transported from the ER to the cytosolic side of the cis-Golgi apparatus membrane by a CERT-independent mechanism (25,26). Here a transmembrane glycosyl transferase, glucosylceramide synthase, catalyses the glycosylation of the primary hydroxyl group in ceramide using UDP-glucose as a donor

glycoside. The glucosylceramide synthase has its catalytic site facing the cytosol where newly produced glucosylceramide (GlcCer) can be recognized by lipid transport protein FAPP2 which mediates the non-vescicular transport of glucosylceramide to distal Golgi compartments (27,28). GlcCer then flips into the Golgi lumen, where the addition of the next sugar residue leads to the formation of lactosylceramide (LacCer). GalCer and GlcCer are precursors of the hundreds of known glycosphingolipids, which are formed by the sequential transfer of sugars by galactosyltransferases, sialyltransferases, GalNAc transferases and GalCer sulfotransferases, all of which are located in the Golgi apparatus (29). The final orientation of glycosphingolipids during biosynthesis is consistent with their nearly exclusive appearance on the outer leaflet of the plasma membrane. Although ceramide resides on intracellular organelles such as mitochondria, glycosphingolipids beyond GlcCer are not known to exist on membranes facing the cytoplasm (30,31). The biosynthesis of glycosphingolipids in the brain provides an example of how competing biosynthetic pathways can lead to glycan structural diversity (32). In the brain, stepwise biosynthesis of GalCer and sulfatide occurs in oligodendrocytes, the cells that elaborate myelin. During oligodendrocyte differentiation, sulfatide is first detected at the stage of immature oligodendrocytes and is upregulated before cells wrap myelin around axons, suggesting that sulfatide not only fulfills a role as a structural component of myelin (33). Gangliosides, in contrast, are synthesized by all cells, with concentration of the different forms varying according to cell type.

During development the expression patterns of glycosphingolipids shifts from simple gangliosides, such as GM3 and GD3, to complex gangliosides, such as GM1, GD1a, GD1b, and GT1b. This shift is regulated primarily by the differential expression and intracellular distribution of the enzymes required for the biosynthesis of the glycosphingolipids (34). In some cases, multiple glycosyltransferases compete for the same glycosphingolipid precursor (35). For example, two enzymes accept LacCer as a substrate: sialyltransferase I that forms ganglioside GM3 and GalNAc-transferase that forms glycolipid GA2 even though for different reasons, including different enzyme kinetic constants and enzyme and substrates cellular localization, much more GM3 is formed than GA2 (21,36,37). Another example, the ganglioside GM3 may be acted on by N-acetylgalactosaminyltransferase, thereby forming GM2, the simplest of the “a-series” gangliosides, or by sialyltransferase II, thus forming GD3, the simplest of the “b-series” gangliosides (38,39). Since sialyltransferases cannot directly convert a-series gangliosides to their corresponding b-series gangliosides, each branch is considered a

committed pathway. Due to this branch exclusivity, the relative expression level of the final glycosphingolipids products is determined by the competition of two enzymes at a key branch point. The transfer of N-acetylgalactosamine to a-, b-, and c-series gangliosides, transforming GM3 into GM2, GD3 into GD2, or GT3 into GT2, is catalysed by the same N-acetylgalactosaminyltransferase. Likewise, the transfer of galactose to GM2 to form GM1, to GD2 to from GD1b, or to GT2 to form GT1c is accomplished by a single galactosyltransferase (40). An additional level of regulation may occur via stable association of different glycosphingolipid glycosyltransferases into functional "multiglycosyltransferase" complexes. The enzymes involved are thought to act concertedly on the growing glycosphingolipid without releasing intermediate structures, ensuring progression to the preferred end product (41).

The breakdown of glycosphingolipids is a stepwise process that occurs predominantly in endosomes and lysosomes. Glycosphingolipid-rich membrane parts are internalized and fuse with early endosomes. Here, glycosphingolipids destined for degradation are sorted through formation of multivescicular bodies which reach the lysosomes. After the fusion with the primary lysosomes, glycosphingolipid become exposed to lysosomal hydrolases and, *in vivo*, they are eventually broken down to their individual components, which are then available for reuse (42,43). Metabolism of endocytosed glycosphingolipids is not restricted to the lysosomes. In fact, a limited amount of GlcCer derived from the degradation of complex glycosphingolipids may escape further lysosomal degradation and reenter the glycosphingolipid pathway (44). In addition, direct metabolic remodeling of glycosphingolipids at the plasma membrane may result in local formation of simpler glycosphingolipids from complex ones (45).

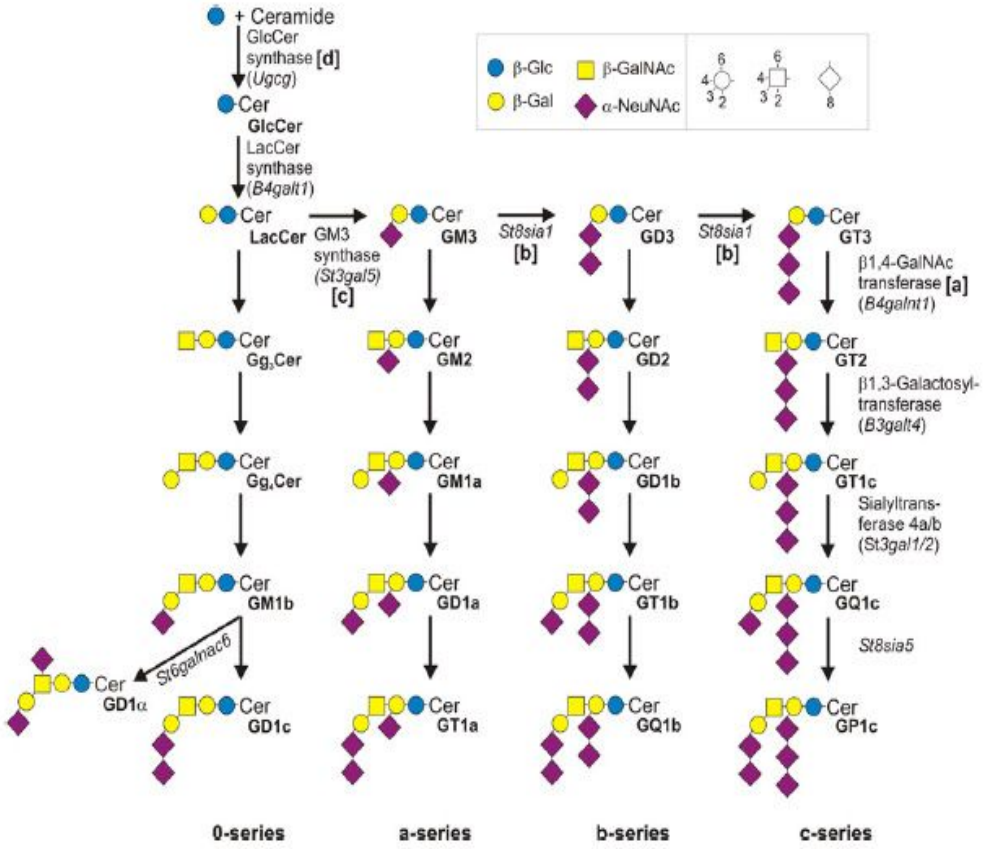


Figure 2. Biosynthetic pathway of ganglio-series derived GSLs. Glycosphingolipids are abbreviated according to the recommendations of the International Union of Pure and Applied Chemistry - International Union of Biochemistry Joint Commision on Biochemical Glycolipids (Jennermann, R. et al., 2013).

Biological functions of glycosphingolipids

Glycosphingolipids are essential for the survival, proliferation, and differentiation of eukaryotic cells within complex multicellular systems. Several studies involving the analysis of genetically engineered mice deficient in gangliosides synthases have revealed the vital importance of glycosphingolipids in the life of the cells that are dealing with a multifaceted extracellular reality. Glycolipid-deficient cells, such as the CM-95 mutant melanoma cell line, lacking glucosylceramide synthase activity (46), and embryonic stem cells from glucosylceramide synthase knockout mice (47) are able to

survive, grow, and undergo through *in vitro* differentiation. However, glucosylceramide synthase knockout mice are embryonic lethal, and showed no cellular differentiation beyond the primitive germ layers (48). Mice lacking all ganglioside expression resulting from knockout of both GalNAcT and SAT-I genes suffer severe lethality. These ganglioside-deficient mice reveal enhanced cell apoptosis, axonal degeneration and perturbed axon-glia interactions (49). It still remains to be cleared whether those phenotypes result from functional deficiency of the particular ganglioside product or from an acquired consequence of the accumulation of substrate precursors.

As mentioned in the previous sections, glycosphingolipids are not randomly distributed along the membrane surface, but they are highly segregated together with cholesterol in lipid domains with specialized signaling functions (50). The glycosphingolipids are usually highly asymmetrically enriched in the external leaflet of the plasma membranes, with the oligosaccharide chain protruded toward the extracellular space, where the sugar residues can engage *cis* and *trans* interactions with a wide variety of cell surface and extracellular molecules (51). These interactions are influenced by the local concentration of glycosphingolipids in the plasma membrane. In the case of *trans* interactions, it has been shown that recognition of lipid-bound oligosaccharides by soluble ligands (for example antibodies or toxins) or by complementary carbohydrates and by carbohydrate binding proteins (such as selectins or lectins) belonging to the interfacing membrane of adjacent cells is strongly affected by their degree of dispersion (or segregation) (52). On the other hand, direct lateral interactions (*cis* interactions) with plasma membrane proteins or short range alterations of the lipid microenvironment of plasma membrane proteins, are strongly favored within a sphingolipid-enriched membrane domain (53). There is a large body of data showing roles for glycosphingolipids as antigens, as mediators of cell adhesion, through *trans* interactions, and as modulators of signal transduction and the list of functions of glycosphingolipids is extensive. Administration of exogenous gangliosides dissolved in the culture medium is a widely used experimental model for the study of these functions in intact cells or in membrane preparations. The binding, uptake and metabolic fate of exogenous gangliosides under different experimental conditions have been well characterized (44,54). The addition of exogenous gangliosides resulted in the modulation of the biological activity of tyrosine kinase receptors, protein kinases and phosphatases, ion channels and pumps, and in cultured neurons and neurotumoral cell lines, it exerted neuritogenic, neurotrophic, and neuroprotective effects (34,55,56). Evidence of the role

of glycosphingolipids as receptors was found studying the internalization of bacterial toxins. For example, GM1 acts as a receptor for cholera toxin B subunit (57), whereas Gb3 acts as a receptor for Shiga toxin and verotoxin (58,59). Also two of the surface proteins of the influenza virus are specifically aimed against the terminal Neu5Ac group on the glycosphingolipids and glycoproteins of the human host (60).

Many pieces of evidence indicated that sphingolipid biosynthesis is necessary for the differentiation and function of neurons in culture. Pharmacological inhibition of glycosphingolipid biosynthesis by synthetic inhibitors of glucosylceramide synthase (such as D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, D-PDMP) (61), or by inhibitors of sphinganine N-acyltransferase (the enzyme that catalyses the synthesis of the biosynthetic precursor of ceramide) (62), reduced axonal elongation and branching in cultured hippocampal and neocortical neurons (63,64). Conversely, up-regulation of glycosphingolipid biosynthesis by L-PDMP stimulated neurite outgrowth in cultured cortical neurons (64). In the same cellular model, D-PDMP and L-PDMP also exerted opposite effects on the formation of functional synapses and on synaptic activity (65).

The role of gangliosides in the maintenance of neuronal structure and function can be at least in part explained by their ability to laterally interact with specific proteins at the level of the plasma membrane and to modulate their activity. Possible interactions with functional significance between gangliosides and plasma membrane proteins have been intensively studied in the past (66-68). Glycosphingolipids are known to interact with growth factor receptors, to modulate cell growth, and in many cases to inhibit receptor-associated tyrosine kinases. Well studied examples of these interactions are represented by epidermal growth factor receptor (EGFR), whose tyrosine phosphorilation and dimerization are inhibited by GM3, but uninfluenced by GM1 (69), and by insulin receptor, inhibited by GM3 but not GD1a (70). On the other hand, there is evidence suggesting that overall lipid raft dynamics, as determined by their peculiar composition, might be rather responsible for the functional modulation of raft-associated signaling proteins (17,71,72).

Glycosphingolipids and cancer

Glycosphingolipids play important roles in modulating several properties of tumor cells. Most tumor cells show altered glycosphingolipid patterns on their surface as well as

abnormal sphingolipid signaling and increased glycosphingolipid biosynthesis, which together play a major role in tumor growth, angiogenesis, and metastasis (73,74). For example, the human sialidase Neu3, which is found on the plasma membrane in *caveolae*-containing microdomains and cleaves terminal Neu5Ac residues from glycosphingolipids, is overexpressed in many types of cancer and plays an important role in tumor growth and survival (75). Malignant transformation is also associated with abnormal glycosilation, resulting in the synthesis and expression of altered carbohydrate determinants, including those on glycosphingolipids. A correlation between the expression of some of these carbohydrate determinants and tumor patient survival rates has been observed, and elevated serum ganglioside levels have been reported in patients (74). On the other hand, in tumor cell lines, the tumorigenic potential correlates with the cellular levels of gangliosides (76,77), and the ability to form experimental tumors can be affected by the artificial manipulation of cellular gangliosides levels (78). The contribution of transformation-associated changes in glycosphingolipids composition to the tumor phenotype is very complex and likely implies heterogeneous molecular mechanisms. However, at least two well-established paradigms support this role: 1) gangliosides have been described as modulators of growth factor receptor function-associated tyrosine kinase activities and cellular compartmentalization. A well-studied example is represented by the interaction between GM3 ganglioside and the EGFR. GM3 negatively regulates ligand-stimulated autophosphorylation and dimerization of EGFR (79-82), and cross-talk of EGFR with integrin receptors (83) and PKCα (84), inhibiting EGFR-dependent cell proliferation and survival in neoplastic cells. 2) GM3 and/or GM2 inhibit integrin-dependent tumor cell motility via the formation of a ganglioside/tetraspanin/integrin receptor complex (the "glycosynapse") that is responsible for the negative regulation of c-Src (85) and Met (86,87) tyrosine kinase activity. Indeed, altered GM3 ganglioside expression plays a multiple role in the control of tumor cell motility, invasiveness, and survival. GM3 is highly expressed in non-invasive compared with invasive bladder tumors and derived cell lines (88,89), and the overexpression of GM3 synthase reduced cell proliferation, motility, and invasion in mouse bladder carcinoma cells (78). In colorectal (90) and bladder (89) cancer cells, GM3-mediated inhibition of integrin-dependent cell motility required the expression of hydrophobic membrane adapter proteins belonging to the tetraspan membrane protein superfamily (tetraspanins). In bladder cancer cells characterized by high GM3 levels and by the expression of tetraspanin CD9, a CD9/α3 integrin complex was stabilized by

GM3-mediated interactions, and the Src C-terminal kinase Csk was recruited to this complex, with consequent inhibition of c-Src and reduced cell motility (85). On the other hand, tetraspanin CD82 is essential for the ganglioside-mediated cross-talk of EGFR with other signaling pathways (84). Thus, a crucial aspect in the control of receptor function by GSL is represented by their ability to influence the formation of multimolecular complexes that usually require the presence of hydrophobic membrane proteins as scaffold or molecular organizers.

Glycosphingolipids are also involved in drug resistance. The effectiveness of many chemotherapy agents and radiotherapies in treating cancers has been found to rely on their ability to increase levels of ceramide in tumor cells so as to activate ceramide-mediated apoptosis. Many tumors have increased expression and activity of glucosylceramide synthase. Drug resistant cancer cell lines show up to threefold higher levels of glucosylceramide. Many tumors also achieve drug resistance by actively pumping out the drugs through the family of ABC transporter proteins. Overexpression of the most common of these efflux pumps, P-glycoprotein, coincides with abnormally high glucosylceramide synthase activity in multidrug-resistant breast cancer, leukemia, melanoma, and colon cancer. P-glycoprotein is a plasma membrane anchored protein that is situated in GSL-containing membrane microdomains. Through the consumption of ATP it is capable of transporting a wide range of noncharged amphiphilic molecules, including glucosylceramide, from the cytosol to the outer plasma membrane. Overexpression of the P-glycoprotien efflux pump is actually one of the most consistent hallmarks of drug resistance (91,92).

Caveolin-1

Caveolin-1 structure, localization and function

Caveolins (93,94) are a family of 21- to 24-kDa integral membrane proteins originally described as the main structural component of *caveolae*, omega-shaped invaginations of the plasma membrane that form a subdomain of cholesterol- and sphingolipid-rich lipid rafts and that are morphologically distinct from the triskelion structure of clathrin-coated pits (16,95). *Caveolae* are decorated by a striated coat that is recognized by antibodies directed against caveolin-1. Caveolin-1 is a key structural component of *caveolae* and its expression is essential for *caveolae* biogenesis (93). Currently, three

caveolin genes are known to exist. While caveolin-3 is found mainly in skeletal muscle fibers and cardiac miocytes (96), caveolin-1 and caveolin-2 are co-expressed in most cells and share many physical properties, although the latter seems to lack full functional capacity to form *caveolae* (97). Cav1 is a 178 amino acid protein that was first identified as a substrate of v-Src kinase in Rous sarcoma virus-transformed chick fibroblasts (93). *In vivo*, two isoforms of caveolin-1 are known to exist: α-caveolin that contains residues 1-178 and β-caveolin that contains residues 32-178. Therefore, only α-caveolin is able to become tyrosine phosphorylated upon Src transformation (98). More recently, other tyrosine kinases such as c-Abl and Fyn have been shown to phosphorylate Cav1 on its tyrosine 14 residue (99,100). Caveolin-1 consists of hydrophobic N- and C-terminal domains, both of which are cytosolic. Three palmitoylation sites in the C-terminal region next to the transmembrane domain contribute to anchoring caveolin proteins to the membrane. The N-terminal region of all caveolins contains a conserved FEDVIAEP motif that has been defined as the 'caveolin signature' sequence, and has been suggested to be important for the binding of caveolin to cholesterol- and glycosphingolipid-rich membrane domains (101). The central segment of caveolin proteins contains the scaffolding domain, which allows oligomerization of caveolin monomers (forming complex comprised of 14-16 monomers) and direct interaction with other proteins, like Src family tyrosine kinases, growth factor receptors, endothelial nitric oxide synthase, G proteins and G-protein-coupled receptors, presumably regulating their activity (102,103). In addition, caveolin proteins contain several serine and tyrosine residues within their intracellular domains that are substrates for a variety of kinases, and become phosphorylated in response to different stimuli (104,105).

Cav-1 localizes to plasma-membrane *caveolae* and also to the Golgi apparatus and trans-Golgi-derived transport vesicles (106,107). Cav-1 may have a soluble cytoplasmic form, as well as a secreted form, depending on the cell type (108), and the first 31 amino acids may be important in selectively targeting isoforms of Cav-1 to different cellular compartments (109).

As previously stated, caveolin-1 is essential for *caveolae* formation and a threshold level of Cav-1 is required to generate *caveolae* at the plasma membrane (93). Though, it is important to note that caveolin-1 can form functional microdomains at the plasma membrane independently of its ability to form *caveolae* (110). Caveolin-1 also plays an essential role in lipid uptake and regulation (111), transcellular transport and signaling

in endothelial cells (112), but also for the entry of certain viruses into mammalian cells (113,114). The abundance of *caveolae* in endothelial cells led to the characterization of a role for Cav-1 in *caveolae* formation and endocytosis, particularly transendothelial transport (115,116).

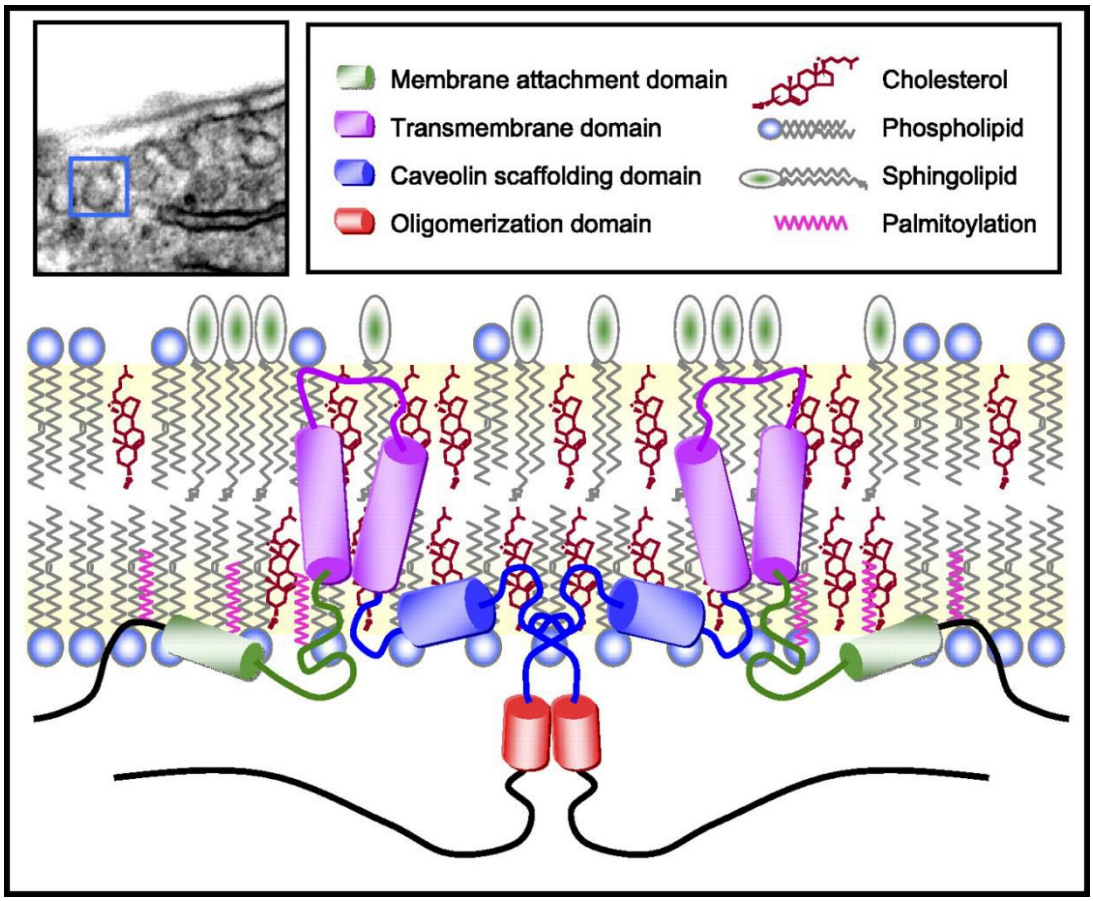


Figure 3. Structure of caveolin-1. Cav-1 contains a hydrophobic central domain with a hairpin-like conformation inserted in the inner leaflet of the plasma membrane. 14-16 Cav-1 monomers form a single cav-1 homooligomer. A simplified dimmer is illustrated here. Both the COOH and NH2 terminus of the cav-1 monomer face the cytoplasm. The caveolin scaffolding domain is located between amino acid residues 82-101 in the NH2-terminal region adjacent to the hydrophobic membrane-insertion domain. The COOH-terminal region of cav-1 contains 3 palmitoylated cysteine residues. *Inset*: caveolae viewed by electron microscopy (×50,000). (From Yang Jin and Augustine M. K. Choi, 2011)

Caveolin-1 and tumor progression

Several pieces of evidence indicate that caveolin-1 influences the development of human cancers. However, the exact functional role of caveolin-1 is still controversial. Caveolin-1 expression increases with cell differentiation. For example, exposure of pre-adipocytes to differentiation-inducing agents induces caveolin-1 mRNA and protein expression (117). Moreover, in addition to endothelial cells, Cav-1 is highly expressed in terminally differentiated cells such as adipocytes, pneumocytes, chondrocytes and smooth muscle cells. Thus, consistent with its anti-proliferative activity, it appears that cell differentiation is accompanied by increased expression of caveolin-1 and *caveolae* (118). Evidence of an inverse relationship between caveolin-1 expression and transformation was first reported in 1995, with the observation that transformation of NIH-3T3 by Abl and Ras oncogenes led to decreased cellular levels of Cav-1 (119). A few years later a direct link between Cav-1 expression and transformation was established when restoration of caveolin-1 expression in the same Abl- or Ras-transformed NIH-3T3 cells was sufficient to abrogate their anchorage-independent growth (120). In NIH-3T3 fibroblasts, antisense inhibition of caveolin-1 expression is sufficient to induce the oncogenic transformation. It also activates MAPK expression and stimulates anchorage-independent-growth (121). The tumor suppressor effect of caveolin-1 was later confirmed in breast cancer cells (122), in tumorigenic cell hybrids (123), in lung alveolar epithelial cells (124), in squamous cell carcinoma (125), and in transformed fibroblasts in which the c-Myc oncoprotein was found to transcriptionally repress Cav-1 expression (126). The development of Cav-1 depleted knockout mouse models confirmed the “transformation suppressor” function of caveolin-1. Loss of caveolin-1 is required to accelerate tumorigenesis and metastasis: PyMT/Cav-1 (-/-) mice showed accelerated onset of mammary tumors and lung metastasis (127). The human genes encoding Cav-1 were localized to the q31.1-q31.2 region of the chromosome 7 at the D7S522 locus. Interestingly, this locus is frequently deleted in a variety of human cancer types including prostate, breast, renal cell carcinoma, and ovarian adenocarcinomas (128). Also, a sporadic mutation in the caveolin-1 gene, at codon 132 (P132L), has been found in 16% of human breast cancer. This mutation behaves in a dominant-negative manner, explaining why only a single mutated Cav-1 allele is found in patients with breast cancer, and leads to formation of misfolded Cav-1

oligomers that are retained within the Golgi complex and are not targeted to *caveolae* (129).

Although its negative role in transformation has been demonstrated using both *in vitro* and *in vivo* models, there is accumulating evidence that caveolin-1 does not have the same function in all types of tumor. Clinicopathological analysis of human pancreas, esophageal, breast, renal cell, brain, lung, and prostate cancer reveal that Cav-1 upregulation is correlated with reduced survival. However, consistent with the tumor suppressor role attributed to caveolin-1, decreased Cav-1 levels are observed in cancer, relative to normal human breast, lung, ovary, thyroid, and mesenchimal tissues. Furthermore, the association of Cav-1 expression with tumor progression is variable in studies of colon, renal cell and oral carcinomas (118). One way to reconcile the conflicting data is to consider the possibility that the role of caveolin-1 may depend on tumor stage. For example, the pattern of caveolin-1 expression in benign prostatic epithelium and human prostate cancer is consistent with tumor suppressive activity (130). Similarly, Cav-1 expression is reduced in breast tumors relative to normal tissue with only a minority of tumors, mainly representing late, advanced stages, including but not limited to inflammatory breast cancer, showing caveolin-1 positivity and poor prognosis, which most likely is a consequence of hypomethylation of its promoter (131-134).

Several studies also reported a correlation between caveolin-1 expression and metastasis. Expression of caveolin-1 in a highly metastatic carcinoma-derived cell line suppressed lung metastasis *in vivo* and reduced invasion *in vivo*. Decreased invasion in caveolin-1 expressing cells was accompanied by reduction in MMP9 and MMP2 secretion and gelatinolitic activity, and reduced ERK 1/2 signaling in response to growth factors (127). Caveolin-1 potentially restrains tumor cell growth and metastatic potential. Cav-1 re-expression in human breast cancer and in colon carcinoma cell lines inhibited tumor cell growth (122), reduced tumorigenicity (135), negatively affected *in vivo* tumor growth, metastasis development and invasiveness in metastatic mammary tumor cells and promoted cell-cell adhesion in ovarian carcinoma cells by a mechanism involving inhibition of Src kinases (136).

On the other hand, there is increasing evidence to suggest that the caveolins and *caveolae* may also be involved in shifting the tightly regulated balance from anti-apoptotic to pro-apoptotic signaling. Caveolin-1 has been shown to interact and inactivate a number of signaling molecules involved in survival/proliferation, such as

the PDGF receptor and phosphatidylinositol 3-kinase (PI3K) (137-139). However, aside from this seemingly pleiotropic suppression of plasma-membrane-initiated pro-survival pathways, *caveolae* and the caveolins appear to have a highly specialized role as well. Ceramide is an essential factor for commitment to apoptosis induced by several cellular stress factors (140). Interestingly, sphingomyelin, the precursor to ceramide generation, is one of the most abundant lipids in *caveolae* microdomains (141). Furthermore, overexpression of caveolin-1 determines inhibition of PI3K signaling and sensitizes cells to ceramide-induced cell death (139). Therefore, the production of ceramide and its downstream actions seem to depend on caveolar localization and caveolin-1 regulation. In support of these results, overexpression of caveolin-1 sensitizes cells toward apoptotic stimuli, whereas antisense-mediated down-regulation of caveolin-1 imparts resistance to apoptosis (142).

Caveolin-dependent signaling and glycosphingolipids

Caveolin-1 regulates cell fate thanks to its ability to bind various signaling molecules. This observation led researches to propose that caveolins constitute a family of scaffolding proteins that organize "preassembled signaling complexes" at the plasma membrane by locally increasing the concentration of signal transducers and by regulating the activation state of *caveolae*-localized signaling molecules (143). As discussed previously, caveolin-1 and sphingolipid-rich membrane complexes are dynamically interacting and interdependent in their compositional regulation. So far, at least two different mechanisms can be hypothesized to explain the effects of glycosphingolipids on signaling complexes organized by caveolin-1. First, both caveolin-1 and glycosphingolipids are simultaneously required in some cases to organize the molecular architecture of a signaling complex. This seems the case for EGFR: for this receptor the formation of a signaling complex with caveolin-1, tetraspanin CD82 and GM3, probably in noncaveolar membrane regions, allows the interaction of EGFR with activated PKC-α, ultimately leading to the inhibition of EGFR signaling (84,144-147). However, this signaling complex does not seem to require a direct caveolin-GM3 interaction (84). Second, in other cases, caveolin-1 and sphingolipids can compete for a common interactor. This is exemplified by insulin receptor (IR), which can form complexes with caveolin-1 required for insulin signaling leading to the translocation of GLUT4 at the surface of normal adipocytes (148).

Accumulation of GM3 upon acquisition of insulin resistance leads to the displacement of IR from caveolin-1 complex and its sequestration as a complex with GM3 (149). In this case it has been convincingly demonstrated that a direct GM3-IR interaction is required. Increased GM1 cellular levels lead to the displacement of another growth factor receptor, PDGFR, located in *caveolae*, negatively regulating Src mitogenic signaling. However, in this case it is not known whether the formation of a PDGFR-GM1 complex is required for its uncoupling from *caveolae*. Since caveolin-1 can directly bind sphingolipids, it cannot be excluded that in this case GM1 forms a complex with caveolin-1, or that enrichment in GM1 inside the *caveolae* induces a deep reorganization of caveolar membrane, thus excluding PDGFR from *caveolae* (150).

The examples reported above illustrate how caveolin-1 and glycosphingolipids could cooperate or compete in the multimolecular organization of a membrane receptor with its interactors, thus potentially affecting the coupling of the receptor with the downstream signaling events and regulating the receptor activity. Another way to regulate receptor function that can be influenced by both caveolin and sphingolipids is represented by the downregulation of plasma membrane receptor concentration through its internalization. The internalization of plasma membrane components, including basal state, ligand-activated or transactivated receptors, can exploit different routes whose complexity has been only recently and partially unveiled. This usually leads to important consequences for the receptor activity, encompassing its sequestration in intracellular sites, recycling to the plasma membrane, intracellular degradation or translocation to the nucleus. Internalization of receptors occurs via clathrin-dependent and clathrin-independent pathways (151). Both mechanisms are involved in the removal of receptor tyrosine kinases (RTK) from the plasma membrane upon ligand activation. Clathrin-dependent endocytosis represents a single traffic pathway well characterized in its step and molecular aspects. On the other hand, clathrin-independent endocytosis encompasses several different pathways that are much more poorly understood in their molecular mechanisms and physiological significance. Among those, caveolin-1-dependent, cholesterol-sensitive endocytic mechanism is usually referred to as “caveolar endocytosis”. Caveolar endocytosis is dependent not only on caveolin-1, but also on *caveolae*, and endothelial *caveolae* contain the whole array of molecular components for a vesicular transport system (152). Lipid raft-dependent (cholesterol- and sphingolipid-sensitive) but caveolin-1-independent internalization pathways have also been described. This picture is still fragmentary, but it becomes apparent that *caveolae*-

and lipid rafts-dependent endocytosis are similar but distinct processes, that are interdependent and reciprocally regulated (153,154). The situation is made even more complex by two relevant observations: 1) association of a molecule with lipid rafts/*caveolae* does not necessary imply its internalization via a caveolae/lipid raft-mediated pathway. Multiple endocytic pathways have been described for the internalization of lipid raft markers, as well exemplified by the case of cholera toxin, that can be internalized via *caveolae* but also via *caveolae*/raft-independent mechanisms, including clathrin-dependent endocytosis, despite its initial binding to GM1 within lipid raft (155-158); 2) usually the internalization of lipid raft components via the clathrin-mediated mechanism requires that they move outside *caveolae*/lipid rafts compartment. However, in some cases, lipid raft recruitment is an essential prerequisite for clathrin-dependent endocytosis, indicating that the association with lipid rafts can modulate as well lipid raft independent internalization mechanisms (159,160). Under basal conditions, *caveolae* are relatively immobile structures with a low turnover at the plasma membrane levels (161), and are thus probably not heavily involved in constitutive endocytic trafficking. However, *caveolae* (and caveolin) can be mobilized and internalized upon specific stimuli (e.g., antibody mediated cross-linking of GPI-anchored alkaline phosphatases (116) and of major histocompatibility complex class I (MHC I) (16), cell membrane attachment of SV40 virus (114), disengagement of integrin receptors upon cell detachment (162)). Based on the observations that the loss of caveolin-1 does not impair endocytosis of some lipid raft markers (163) and that caveolin-1 levels inversely correlate with the uptake of raft-associated receptors (e.g., reduction of caveolin levels accelerate raft-mediated internalization of autocrine motility factor receptor (164)), it has been proposed that caveolin-1 could indeed act as a negative regulator of *caveolae*/raft-mediated receptor uptake, stabilizing and immobilizing potentially endocytic raft domains (153,154). Triggering of *caveolae*/raft-mediated internalization would thus require additional factors allowing to overcome the restraint to endocytosis imposed by caveolin-1. It has been shown that *caveolae*/rafts internalization in response to specific stimuli is dependent on glycosphingolipids and tyrosine phosphorylation. Sphingolipids are essential for clathrin-independent endocytosis (165), and glycosphingolipids stimulate caveolar endocytosis (166). Glycosphingolipids could directly affect the membrane environment of caveolin-1, or could regulate tyrosine phosphorylation of caveolin-1, that is essential requirement for caveolar/raft endocytosis (162). Indeed, using fluorescent sphingolipids analogues it has

been proven that sphingolipid segregation in endocytic vesicles is essential for caveolar endocytosis. On the other hand, glycosphingolipids-stimulated caveolar endocytosis required Src activity, and addition of exogenous sphingolipids or cholesterol has been shown to stimulate cholesterol activity (167). Thus, likely multiple mechanisms regulated by sphingolipids are potentially responsible for triggering caveolar endocytosis.

Along this line, another mechanism that could be involved in glycosphingolipid-regulated and caveolin-mediated clearance of plasma membrane receptors is suggested by the observation that many RTKs are at least in part localized in lipid rafts, have a caveolin binding motive and form complexes with caveolin-1. In all these cases, the elevation of cellular gangliosides levels has as a consequence the shift of the receptor outside of *caveolae*. In the case of IR and PDGFR, this results in the uncoupling of the receptor from the downstream signaling cascade. However, as mentioned above, movement of RTKs outside caveolar membrane domains potentially target these receptors to clathrin-dependent internalization pathways, thus contributing in the negative regulation of the cell surface concentration of the receptor.

Src kinases

Src tyrosine kinases

Tyrosine kinases play important roles in controlling animal-specific cellular functions such as rapid cell-cell communication via the plasma membrane. The most critical feature of tyrosine kinases is the strict regulation of their functions. Due to these enzymes' importance in controlling cellular function, dysregulation of tyrosine kinases can play a causative role in various diseases, especially cancers. Tyrosine kinases are classified into two major subgroups: receptor and non-receptor. The Src family tyrosine kinases (SFKs) are the major non-receptor tyrosine kinases expressed in multiple types of animal cells and are involved in many of the signaling mechanisms associated with G-protein-coupled receptors, integrins, receptor tyrosine kinases, T-cell receptors, and others (168). The members of the Src family tyrosine kinases include Src, Yes, Fyn, Lyn, Blk, Fgr, Hck, and Lck. Of the members of the family, c-Src, Yes, and Fyn are expressed ubiquitously, with the other members being expressed primarily in lymphocytes (169). SFKs all share a common general structural organization: an N-

terminal membrane association domain, a unique domain, a Src homology (SH) 3 domain, an SH2 domain, a catalytic domain, and a C-terminal regulatory domain. The N-terminal domain contains signals for lipid modification: myristoylation (in all SFKs) and palmitoylation (in all but c-Src and Blk) signals, both of which are required for membrane association of SFKs. In particular, palmitoylation has been implicated in the stable localization of SFKs in lipid rafts (170). All SFKs share the SH3 and SH2 domains, the catalytic domain and the C-term tail. The catalytic domain contains an autophosphorylated tyrosine (Y416 in c-Src), which is phosphorylated when the enzyme is active. SH2 domains bind phosphotyrosine motifs, and SH3 domains bind prolin-rich motifs. In the inhibited state, the SH2 domain of c-Src is involved in an intramolecular interaction with a C-terminal regulatory domain phosphotyrosine (Y527) locking the enzyme in an inactive or closed state (171). In the case of v-Src, oncogenic activation results from the loss of this C-terminal regulatory domain (172). Phosphorylation of the C-terminal regulatory tyrosine is catalysed by C-terminal Src kinase (Csk) (173). Csk knockout mice die at embryonic day 9 or 10, therefore it has been hypothesized that Csk might be required for normal development (174). The structure of Csk is similar to SFKs with an SH2 domain, an SH3 domain, and a kinase domain. It is known that Csk lacks a regulatory C-terminal tyrosine, N-terminal myristoylation, and membrane association domain (173). Although SFKs are membrane-associated and regulated by phosphorylation, Csk is intrinsically cytoplasmic and requires membrane adaptors to inhibit membrane-associated SFKs (175). On the other hand, in the case of c-Src, activation of growth-factor receptors leads to their association with the SH2 domain, which disrupts inhibitory intramolecular interactions to promote c-Src activation. Other proteins, such as CRK-associated substrate (CAS) and FAK, bind to the c-Src SH2 and SH3 domains to promote c-Src activation by a similar mechanism. Levels of c-Src protein are also negatively regulated by the E3 ubiquitin ligase CBL, which leads to c-Src ubiquitylation and subsequent degradation by the proteasome (176).

The localization of c-Src within the cellular infrastructure is important. The association of c-Src with the plasma membrane is considered essential for cellular transformation (177), and the autophosphorylation of Tyr419 that occurs with membrane targeting, which is enabled by interactions with activated receptor tyrosine kinases, is associated with the highest level of c-Src transforming activity (178). Inactive c-Src is localized at perinuclear sites, but c-Src activation causes its SH3 domain to become indirectly associated with actin. Activated c-Src is ultimately translocated to the cell periphery to

sites of cell adhesion, where it attaches to the plasma membrane inner surface through its myristoylated domain (177). This tethered location allows for interactions with membrane-bound receptor tyrosine kinases and integrins associated with adhesion functions. The localization of c-Src at the membrane-cytoskeletal interface in focal adhesions, lamellipodia and filopodia seems to be regulated by RhoA, Rac1 and Cdc42 (179).

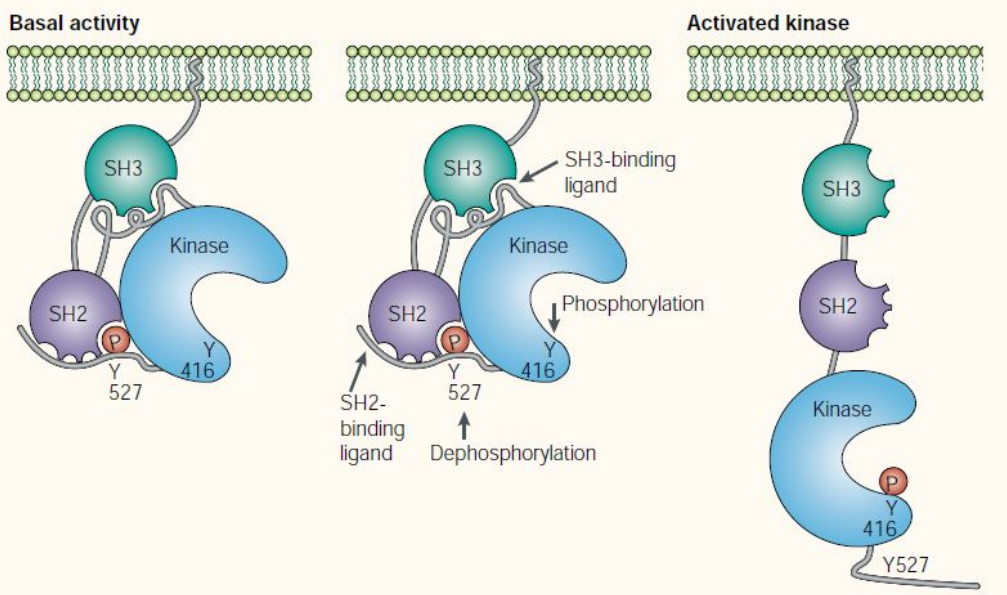


Figure 4. Proposed model for Src activation. The left panel represents the inactive conformation of Src, in which Tyr 527 interacts with the SH2 domain, positioning the SH3 domain to interact with the linker between the SH2 and catalytic domains. The middle panel illustrates different mechanisms involved in the activation of Src, and the right panel represents the open or active conformation (Martin, G.S., 2001).

Src family kinases and tumor

Because SFKs are pleiotropic kinases involved in many cellular events, it is not surprising that aberrant activation of Src signaling contributes to diverse aspects of tumor development (176). SFKs are important mediators of tumor cell proliferation and survival. The most prominent and well-studied function of Src is its extensive interaction with transmembrane receptor tyrosine kinases (RTKs) at the cell membrane via its SH2 and SH3 domains (180). Src has long been known to interact with epidermal

growth factor receptor (EGFR) (181), human epidermal growth factor receptor 2 (HER2) (182), platelet-derived growth factor receptor (PDGFR) (183), and insulin-like growth factor receptor (IGF-1R) (184). Through these interactions, Src integrates and regulates RTK signaling and directly transduces survival signals to downstream effectors such as PI3K, Akt and signal transducers and activator of transcription 3 (STAT-3) (185). Src can also be activated by other membrane receptors including integrins and erythropoietin receptor (EpoR). Src is also known to be crucial during tumor metastasis, mainly as a result of its role in regulating the cytoskeleton, cell migration, adhesion and invasion (186). Through interaction with p120 catenin, Src activation promotes dissociation of cell-cell adherens junction and facilitates cell mobility (176). Through phosphorylation of focal adhesion kinase (FAK), Src activation also stabilizes focal adhesion complexes, which consist of FAK, paxillin, RhoA and other components, and enhances cell adhesion to extracellular matrix (186). Additionally, Src also plays a part in regulating the tumor microenviroment. Under hypoxic conditions, Src activation promotes angiogenesis through stimulation of vascular endothelial growth factor (VEGF) (187), matrix metallopeptidases and interleukin-8 (IL-8) expression (188).

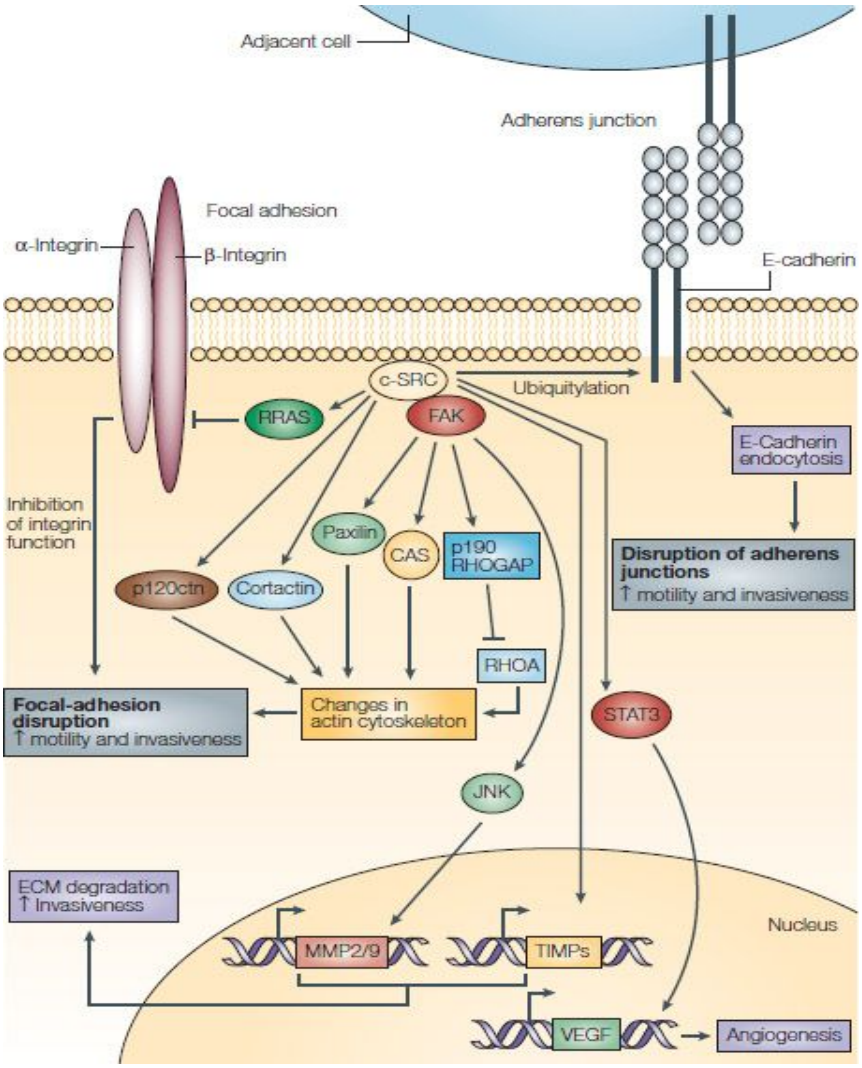


Figure 5. Effects of c-Src on tumor cell behavior. c-Src exerts its effects on tumor-cell behavior through a range of mechanisms mediated by interactions with various substrates and binding partners. A selection of these mechanisms is illustrated here. c-Src promotes motility and invasiveness stimulating E-cadherin ubiquitylation and its subsequent endocytosis, and also by stimulating turnover of focal adhesions in various ways. The binding and activation of FAK, which phosphorylates substrates such as paxillin, CAS and p190 Rho-GAP bring about changes in the cytoskeleton that lead to focal-adhesion disruption. c-Src also brings about similar changes independently of FAK through its interactions with cytoskeleton-associated proteins such as p120 catenin and cortactin. Ras phosphorylation by c-Src inhibits integrin function, which also leads to focal adhesion turnover. c-Src activity also leads to changes in the expression of several genes that contribute to tumor progression. Activation of FAK stimulates the c-Jun N-terminal kinase (Jnk) signaling pathway, leading to increased expression of

the matrix metalloproteinases MMP2 and MMP9; c-Src also induces the expression of various tissue inhibitors of metalloproteinases (TIMPs). MMPs promote the breakdown of the extracellular matrix. STAT3 by c-Src leads to increased expression of VEGF, a signaling molecule that promotes tumor angiogenesis (Yeatman, T., 2004).

Caveolin-1 and c-Src

It has been shown that caveolin-1 promotes cell-cell adhesion in ovarian carcinoma cells by a mechanism involving inhibition of Src kinases (189). Non-receptor tyrosine kinases of the Src family are involved in several cell functions such as mitogenic response of growth factors, fibroblasts cell migration and epithelia cells scattering in cancers (168). Src kinases, located at the inner face of membranes, segregate in the specific membrane domains defined by sphingolipids, and usually enriched in caveolin. Src kinase localization in *caveolae* and/or sphingolipid-enriched domains seems to be instrumental for growth factor-induced Src dependent mitogenic response (170,177). Src kinase are activated and involved in cancer progression and metastasis of most human carcinoma. Publishes results from our group show that c-Src is in a less active state in low-motility human ovarian carcinoma cell lines expressing high levels of GM3 and caveolin-1 (4). Remarkably, it has been demonstrated that Src kinases are activated in colon cancers despite the expression of the C-terminal Src kinase, Csk, the main negative regulator of c-Src and other related kinases (190). Csk is a cytosolic enzyme that may need an intermediary protein to locate in Src kinases vicinity. Several candidates have been described such as paxillin, Csk binding protein/phosphoprotein associated with glycosphingolipids (Cbp/PAG), and caveolin-1. When Cbp/PAG is phosphorylated on tyrosine 314 by active SFKs, Csk is recruited to lipid rafts via binding to Cbp at pY314 and phosphorylates tyrosine 527 to inactivate the catalytic activity of SFKs. The inactivated SFKs then relocate to non-raft compartments (173). Cbp/PAG is downregulated in metastasis. Cbp/PAG could play a major role in Src kinases regulation and cancer progression either as a Csk binding protein or as a glycolipid interacting protein. On the other hand, interactions of Src with caveolin-1 have important consequences. Caveolin-1 seems to act as a membrane adapter which couples integrin receptor to Src kinases (191). Src induces phosphorylation of caveolin-1 at tyrosine 14, which is responsible for the rearrangement of caveolin-1 within the cell

(105,192). On the other hand, caveolin-1 phosphorylation is involved in the regulation of the docking of Csk, the negative regulator of Src, suggesting a mechanism of negative regulation of Src activity by phosphorylated caveolin (193). Moreover, phosphorylated caveolin is recruited to lipid-enriched membrane domains upon integrin receptor disengagement, inhibiting the internalization of these specialized membrane areas and the signaling events downstream to integrin receptor (194-196).

Integrins

Integrin structure

Cells make extracellular matrix, organize it, and degrade it. The matrix in its turn exerts influences on the cells through transmembrane cell adhesion protein that can act as matrix receptors and tie the matrix to the cell cytoskeleton. However, their role goes far beyond simple passive mechanic attachment. In fact, cell adhesion to the extracellular environment has an essential role in cell survival, growth and migration. Although several types of molecules can function as matrix receptors or co-receptors, including transmembrane proteoglycans, the principal receptors on animal cells for binding most extracellular matrix proteins are the integrins.

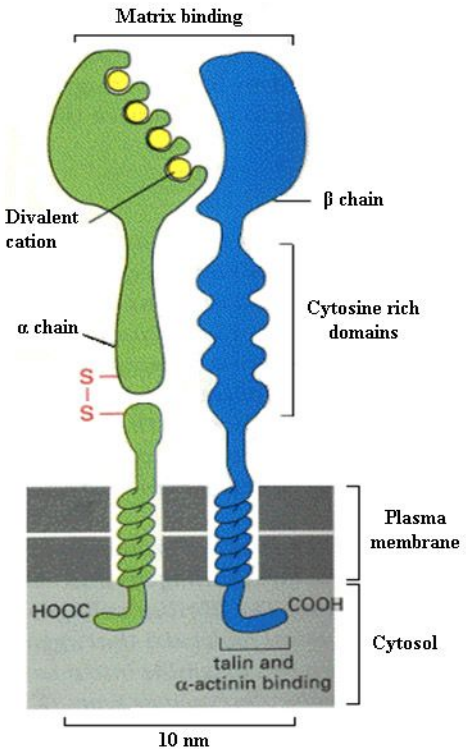


Figure 6. The subunit structure of integrin. Electron micrographs suggest that the molecule has approximately the shape shown here, with the globular head projecting more than 20 nm from the lipid bilayer. By binding to a matrix protein outside the cell and to the actin cytoskeleton inside the cell, the protein serves as a transmembrane linker. The α and β subunits are held together by noncovalent bonds. The α subunit is made initially as a single polypeptide chain, which is then cleaved into one small transmembrane domain and one large extracellular domain that contains four divalent-cation-binding sites; the two domains remain held together by a disulfide bond. The extracellular part of the β subunit contains a single divalent-cation-binding site, as well as a repeating cysteine-rich region, where intrachain disulfide bonding occurs.

Integrins are a family of glycosylated, heterodimeric transmembrane adhesion receptors that consist of noncovalently bound α and β subunits (197). In vertebrates, 18 α subunits and 8 β subunits have been discovered, which combine into 24 different heterodimers that recognize overlapping but distinct sets of extracellular ligands (198). Most integrins recognize several ECM proteins. Conversely, individual matrix proteins, such as fibronectin, laminins, collagens, and vitronectin, bind to several integrins (199). At least

eight types of integrins bind fibronectin, and at least five types bind laminin. Because the same integrin molecule in different cell types can have different ligand-binding specificities, it seems that additional cell-type-specific factors can interact with integrins to modulate their binding activity (200).

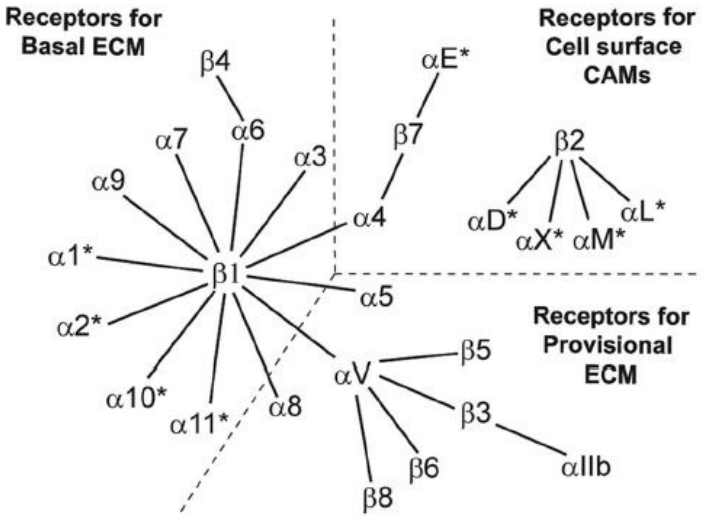


Figure 7. Integrin family. Integrins are loosely grouped into three classes that bind basal extracellular matrix (ECM), provisional ECM and cell surface adhesion molecules (CAMs), respectively. Basal ECM mainly includes collagen and laminin. Provisional ECM mainly includes fibrinogen, fibronectin, vitronectin, cryptic collagen and von Willebrand factor. The α I-containing integrins are asterisked (Zhang, K. et al., 2012).

Through interaction with the ECM, integrins control many cellular processes that occur during development and in the progression of diseases such as cancer. As well as mediating attachment to the ECM, integrins act, directly or indirectly, as bidirectional transducer molecules (201,202). Although they lack any known enzymatic activity of their own, integrins can initiate “outside-in” signaling by recruiting signaling moieties that generate and convey signals to the migratory and proliferative machinery of the cell. Another way in which integrins can convey outside-in signals is through their ability to influence the manner by which growth factor receptor respond to their ligands (203). Integrin function can also be controlled from within the cell through “inside-out” signaling (201). Indeed, integrins can be switched from low-affinity conformation to a high-affinity active conformation through the association of the β-integrin cytoplasmic

tail with proteins such as talin and kindling. This process is important for the coordination of adhesion during the migration of various cell types (204).

Integrin signaling and lipid rafts

Examples of signaling pathways that involve lipid rafts include immunoglobulin E signaling, T lymphocyte activation, glia cell line-derived growth factor (GDNF) signaling and H-Ras mediated Raf activation (17). In all these signaling events, the interaction between the activated receptors and their immediate downstream effectors takes place in the raft fraction of the plasma membrane, and downstream signaling is inhibited by cholesterol depletion. Recent work in this area has focused on the activation of signaling by the small GTPase Rac in response to integrin-mediated cell adhesion to extracellular matrix. Rac is implicated in cell survival, gene expression, cell cycle progression, cell migration, and cell-cell adhesion and its activation by integrins upon fibronectin binding induces GTP loading, similar to the activation triggered by growth factor receptors; but, distinct from growth factor regulation, integrins also target Rac to specific plasma membrane microdomains, where Rac can interact with its downstream effector molecule PAK to induce signaling (205,206). Thus, when the fibronectin binding integrins are uncoupled from downstream signaling by detaching cells from the ECM, PAK is not activated by Rac, even though Rac-GTP is still present in these detached cells (206). These data suggests that integrin-mediated adhesion facilitates the coupling of Rac to PAK by modulating the plasma membrane so as to target Rac to specific microdomains, where the interaction with its effector can take place. There is strong evidence that the membrane microdomain targets of integrin-modulated Rac affinity are lipid rafts or cholesterol-enriched membrane microdomains (CEMM). Not only the association of Rac with CEMM has been reported in several studies (162,207,208), it has also been reported that the loss of integrin signaling promoted by cell detachment induces a rapid CEMM internalization mediated by *caveolae*, and this prevents the targeting of Rac to the plasma membrane and its coupling to PAK (162). These data suggest a model in which integrin-mediated cell adhesion promotes plasma membrane localization of Rac and its subsequent coupling to its effector by preventing internalization of the Rac-containing CEMMs (162). Evidence for association with CEMMs in an integrin-dependent manner has also been reported for the other two members of the Rho family of GTPases, Rho and Cdc42 (208-210).

Rho and its effectors regulate microtubule stabilization, and this process occurs only at the leading edges of migrating cells, which are enriched in Rho and CEMMs. The local coupling between Rho and its effector is regulated by integrin-mediated cellular adhesion to the ECM, and appears to require active signaling by focal adhesion kinase (210). Cdc42 targeting to the plasma membrane is also integrin-dependent (205).

Glycosphingolipids and integrins

One mechanism by which glycosphingolipids could affect cell adhesion and migration is via their interaction with integrins. Glycosphingolipids have been shown to directly modulate integrin-based cell attachment. For example, gangliosides enhance binding of integrins to the ECM in mouse mammary carcinoma, melanoma, and neuroblastoma cells (211-213). Several models have been proposed for the mechanisms by which glycosphingolipids or glycosphingolipid-enriched microdomains may regulate integrin function (214,215). First, glycosphingolipids could initiate signaling events, which cause downstream activation of integrins. Indeed, addition of exogenous glycosphingolipids to cells has been shown to have significant effects on signaling cascades. Another possibility is that glycosphingolipids promote clustering of integrins in glycosphingolipid-enriched microdomains, thus increasing their avidity for ligand. The cross-linking of integrins with certain antibodies is an established method for integrin activation (216,217). Similarly, integrin function can be modulated by antibody cross-linking of cholera toxin B subunit bound to GM1 or GPI-linked proteins (214,215). However, no studies have provided direct evidence that glycosphingolipids modulate integrin clustering in glycosphingolipid-enriched microdomains in the absence of cross-linking agents. An additional mechanism by which glycosphingolipids could regulate integrins is by affecting their endocytosis from the plasma membrane. Recent studies have shown that some integrins can be internalized via *caveolae* (217,218), a subset of glycosphingolipid-enriched microdomains defined as invaginations at the plasma membrane enriched in caveolin-1 (16,95). *Caveolae* are sites for clathrin-independent endocytosis of glycosphingolipids as well as some viruses and bacteria toxins (42,114,166,167). When cells are attached to the ECM, integrins negatively regulate *caveolae* internalization, preventing uncoupling of signaling molecules such as Rac disengagement from its effector PAK. It has been reported that at least three integrin-dependent growth pathways (Ras/Erk, PI3K/Akt, Rac/PAK) are

impaired by Cav-1 mediated CEMM internalization. The presence of caveolin thus links integrin to growth-regulatory pathways; alterations of this regulatory mechanism in the absence of caveolin would uncouple integrins from growth-regulatory pathways and therefore break the requirement of integrins for active signaling, resulting in anchorage independent growth, characteristic of most tumor cells. In addition integrins regulate the focal adhesion localization of tyrosine phosphorylated caveolin-1, preventing its recruitment to *caveolae* and internalization (194,219).

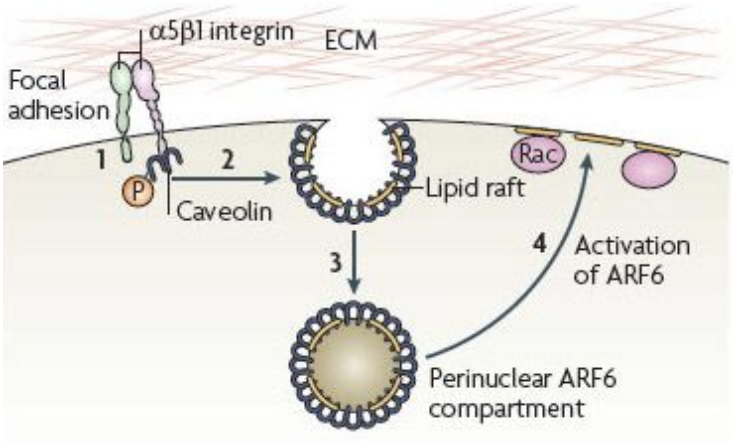


Figure 8. Integrins and *caveolae*. When cells are adherent, integrin engagement with fibronectin leads to the retention of phosphorylated caveolin in focal adhesions (1), which opposes the endocytosis of lipid rafts. Cell detachment triggers the release of phosphorylated caveolin from focal adhesions (2), thus allowing its association with *caveolae* to induce the endocytosis of lipid rafts (3). Re-engagement of α5β1 integrin (1) reverses this process, both by sequestering phosphorylated caveolin to shut down lipid raft internalization and by activating ADP-ribosylation factor 6 (ARF6) to promote rapid recycling of lipid rafts, which recruits active Rac to the plasma membrane (4) (Caswell, P. et al., 2009).

It was also reported that the addition of glycosphingolipids or cholesterol to the plasma membrane of cells stimulates caveolar endocytosis via activation of Src kinase (166). After treatment with exogenous sphingolipids the cells began to reorganize their actin cytoskeleton and retract, suggesting a link between the plasma membrane glycosphingolipid and cholesterol composition and cell adhesion via integrins.

AIM OF THE STUDY

A growing number of evidence suggests that alterations in glycosphingolipids expression and metabolism are common in tumors of different origins. Moreover, gangliosides, sialic acid-containing glycosphingolipids, are known to modulate several cellular functions relevant to tumor progression. Thus, altered ganglioside expression might play a relevant role in determining the aggressiveness and metastatic potential at least in certain tumors. Cellular ganglioside levels deeply affect tumor cell adhesion, motility and migration. In particular, gangliosides might contribute to the modulation of integrin-dependent interactions of tumor cells with the extracellular matrix as well as with host cells present in the tumor microenvironment. Therefore, the influence of gangliosides on tumor cell adhesion and motility seems to be mediated by the regulation of membrane-associated signaling complexes. Gangliosides interaction with hydrophobic membrane adaptor proteins seems to be crucial for this regulation. From this point of view, the interaction between gangliosides and the integral membrane protein caveolin-1 is potentially very interesting. Caveolin-1 is usually highly expressed in terminally differentiated cells, while it is markedly down-regulated in tumors of different origin, including ovarian, breast and colon carcinoma. It has been recently demonstrated that in human ovarian cancer cells overexpressing GM3 synthase, an enzyme involved in the synthesis of complex glycolipids, invasiveness is reduced. It has been shown that in cells with high levels of GM3 synthase, a complex organized by caveolin-1, a hydrophobic membrane protein first identified as the main component of caveolae, regulates cell motility and adhesion by controlling the inactivation of c-Src downstream to integrin receptors.

On the basis of these considerations, the aim of the present study was to investigate the role of gangliosides as modulators of membrane signaling complexes organized by caveolin-1, able to affect the adhesion, motility and invasiveness of human cancer cells, thus contributing to a better understanding of the role of membrane glycolipids in the early mechanisms of the progression and dissemination of human cancer.

MATERIALS AND METHODS

Chemicals

Commercial chemicals were the purest available and, unless otherwise stated, were purchased from Sigma Chemical Co.

Lipids and radioactive lipids

GM1, GM2 and GD1a were prepared from the bovine brain ganglioside mixture and purified by partitioning (220). GM3 was prepared from GM1 using the GM1-lactone hydrolysis procedure (221). [1-^{3}H]sphingosine (radiochemical purity over 98%; specific radioactivity 2.2 Ci/mmol) was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[^{3}H]hydride. [^{3}H] lipids used as chromatographic standards were prepared from [1-^{3}H]sphingosine-fed cell cultures as previously described (227).

Cell lines and culture

Human ovarian carcinoma A2780 cells were kindly provided by Dr. Franca Formelli (*Department of Experimental Oncology, National Cancer Institute*, Milan, Italy) and were obtained by Dr. R.F. Ozols (*National Cancer Institute,* Bethesda, USA). These cells grow as monolayers on an artificial substrate and were cultured in RPMI 1640 medium (Sigma) supplemented with 10% of heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C with 5% of CO_2.

The cell line A2780/HPR, resistant to the drug HPR, was obtained from the parental cells A2780 through *in vitro* administration with increasing sub-lethal concentrations of HPR (Sigma). The cells that survived to 60 selection cycles in HPR containing medium were cloned through limit dilution.

The A2780/HPR clones can survive in presence of HPR at a concentration 10 times higher than the ones used on A2780 cells in the same experimental conditions (222). HPR resistance in these clones is reversible; it was observed that A2780/HPR cells cultured for 5 passages in complete medium without the drug show a marked decrease

in their resistance. For this reason, A2780/HPR cells are grown in complete medium containing 5 μM HPR.

The cell line A2780/SAT-I was obtained by transfection of the parental cells with the cDNA of the sialyltransferase-I (SAT-I) enzyme), also known as GM3 synthase. A2780 cells were transfected with the pRc/CMV expression vector (223), or with an empty vector. The clones stably transfected were isolated using 750 μg/ml of geneticin (G418, Sigma). The A2780/SAT-I cells were cultured in complete medium containing 250 μg/ml of geneticin.

RNA extraction and RT-PCR

Total RNA was isolated by single-step acid-guanidine-isothiocyanate-chloroform extraction methods and purified by PureLink™ Macro-to-Midi kits (Invitrogen), according to manufacturer's instructions. Five micrograms of RNA was treated with 3 U of RNase-free DNase for 25 min at room temperature to remove possible DNA contamination. The total amount of extracted RNA was estimated by a quantitative fluorescent method using the Quant-iT™ RiboGreen RNA Reagent Kit (Invitrogen). Using random hexamers, 1 μg of RNA were reverse-transcribed using SuperScript™ III First-Stand Synthesis System for RT-PCR (Invitrogen) in a final 20 μl reaction volume. cDNA representing 50 ng of total RNA was adapted as a template for RT-PCR.

For multiplex RT-PCR, we used a mixture containing 0.2 μM primers, AccuPrime™ PCR Buffer, and 1 unit of AccuPrime™ Taq in a final volume of 50 μl. The amplification was performed using the following cycle conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 15 s at 94 °C (denaturation), 20 s at 58 °C (annealing) and 30 s at 68 °C (elongation). RT-PCR mixture included 0.5 μM primers, 200 μM deoxynucleotides triphosphate, Fusion HF Buffer, 0.4 U of Phusion Hot Start DNA polymerase (Finnzymes) and 3% of dimethyl sulfoxide in a final volume of 20 μl. The amplification was performed using the following cycle conditions: initial denaturation at 98 °C for 1 min, followed by 30 cycles of 10 s at 94 °C (denaturation), 45 s at 58 °C (annealing) and 30 s at 72 °C (elongation). The housekeeping gene ACTB was used as reaction and loading control and were simultaneously amplified with the target genes. Data were acquired using a GelDoc 2000 instrument (BioRad) and were elaborated using the Quantity One software (BioRad). Primer sequences were summarized in Table 1 shown below.

Table 1: Sequence of primer

Gene	Forward primer	Reverse primer
SAT-I	5'-GGGAGTAATAGCATGGGCAACCAT-3'	5'-CAGCTCTCAGAGTTAGAGTTGCATT-3'
ACTB	5'-CGACAGGATGCAGAAGGAG-3'	5'-ACATCTGCTGGAAGGTGGA-3'

Administration of exogenous gangliosides

A2780 confluent monolayer cells were washed with serum-free culture medium and then incubated in the presence of 50 μM GM3, GM2, GM1 or GD1a in serum-free medium for up to 48 hours [215]. The following experiments were conducted after the treatment.

Glucosylceramide synthase inhibition

To study the effects of ganglioside synthesis inhibition, the GlcCer synthase inhibitor D-PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) was used (221). As negative control, cells were treated with the inactive stereoisomer L-PDMP under the same experimental conditions. D- and L-PDMP were kindly provided by Dr. Jin-ichi Inokuchi (Tohoku Pharmaceutical University, Japan). The compounds were dissolved in distilled water at a concentration of 4 mM. The stock solution was stored at -20 °C and diluted with cell culture medium to a final concentration of 10 μM or 20 μM just before use. A2780/HPR and A2780/SAT-I cells were seeded and cultured in the presence of D- or L-PDMP for 48 h. The effects of PDMP on ganglioside synthesis were detected by analyzing the lipid composition of the treated cells.

In vitro cell motility

Determination of *in vitro* cell motility by wound healing assay

Cells grown in 100-mm culture dishes as confluent monolayers were mechanically scratched using a 200-μl pipette tip or a rubber policeman. Cells were washed with complete culture medium and then incubated in the presence of complete culture medium for different times allowing wound healing. Phase contrast images of the

wounds were taken in nine random fields immediately after wounding (time 0) and after 24 and 48 hours, and wound width was measured. Each experimental point was in duplicate, and data were expressed as the mean values ± S.D. of three independent experiments.

Determination of *in vitro* cell motility by Phagokinetic Gold Sol Assay

Phagokinetic assays with gold colloid-coated plates were performed as described (224). Briefly, 24-mm coverslips were coated with 1% bovine serum albumin (BSA) (Sigma-Aldrich) and then immersed in the colloidal gold solution (225,226). Two-thousand cells were seeded on the gold colloid-coated coverslips and incubated at 37°C. Images of the phagokinetic tracks were taken after different times by the use of a phase contrast microscope. The tracks of at least 50 cells were videocaptured and the areas cleared from gold colloid by cell phagocytosis, representing the migration response, were quantified by the use of Image J software.

Protein analysis

Treatment of cell cultures with [1-^{3}H]sphingosine

To qualitatively and quantitatively analyze the cellular ganglioside expression pattern with high sensitivity, A2780/SAT-I cells were grown in 100 mm diameter Petri dishes and after 24 hours they were incubated with [1-^{3}H] sphingosine that was dissolved in complete culture medium. [1-^{3}H] sphingosine was administered at a concentration of 3×10^{-8} M, which is under the concentration known to cause biological effects. In these experimental conditions the [1-^{3}H] sphingosine administration does not change the viability and the morphology, observed with optical microscopy, of these cells. To perform a metabolic labeling of the sphingolipids at the steady-state, the cells were incubated with the [1-^{3}H] sphingosine containing medium for 2 hours (pulse). After the pulse, the medium was replaced with fresh medium without radioactive sphingosine, and cells were further incubated for up to 2 days (chase). In these experimental conditions the cultured cells efficiently incorporate the radioactive sphingosine that is then quickly acylated to ceramide, which serves as a precursor for all sphingolipids, included the complex ones. With this method of labeling, every sphingolipid (including ceramide, SM, neutral glycolipids, and gangliosides) and phospholipid (obtained by

recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H] sphingosine) were radioactively labeled (227-229).

Preparation of DRM fractions by sucrose gradient centrifugation

Cells were subjected to homogenization and to ultracentrifugation on discontinuous sucrose gradient, as previously described (227). Briefly, cells were harvested, lysed in 1% Triton X-100 in TNEV (10 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA) in the presence of 1 mM Na_3VO_4, 1 mM PMSF, and 75 mU/ml aprotinin, and *Dounce homogenized* (10 strokes, tight). Cell lysate was centrifugated for 5 min at 1300 × g to remove nuclei and cellular debris. The postnuclear supernatant (PNS) was mixed with an equal volume of 85% sucrose (w/v) in TNEV, placed at the bottom of a discontinuous sucrose gradient (30% - 5%), and centrifugated for 17 h at 200,000 g at 4 °C with ultra-centrifuge Beckman Coulter optima L-90K. After ultracentrifugation, eleven fractions were collected starting from the top of the tube. The protein levels and lipid distribution were analyzed individually in each fraction. Moreover, equal amounts of the low-density fractions 4, 5 and 6 were put together to obtain the DRM fraction, whereas equal amounts of the high-density fractions 9, 10 and 11 were put together to obtain the HD fraction. The fractions 7 and 8 were also put together to obtain the intermediate fraction. The entire procedure was performed at 0-4°C in the ice immersion. For investigating the metabolism of sphingolipids and phospholipids, cells were previously labeled with [1-^{3}H]sphingosine.

Caveolin-1 immunoprecipitation

A2780/SAT-I 4T cells were grown in 100 mm diameter Petri dishes. The following day 20 μM D- or L-PDMP were added to the culture medium. A group of cells was left untreated and used as control. The cells were treated for 48 hours with the glucosylceramide synthase inhibitor before proceeding with the experiment.

After the treatment, A2780/SAT-I 4T cells were washed twice with phosphate buffered saline (PBS) 1x containing 1 mM Na_3VO_4, and scraped in PBS-EDTA 0.02% containing 1 mM Na_3VO_4. The cell suspension was then centrifugated at 244 g for 10 minutes at 4 °C, and the cells were resuspended in *lysis buffer* (1% Triton X-100 (w/v), 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA) containing a mixture of protease inhibitors (1 mM Na_3VO_4, 1 mM PMSF, 75 milliunits/ml aprotinin) and homogenized with a *Dounce homogenizer* (10 strokes, tight). Cell lysate was

centrifugated at 1300 g for 5 minutes to remove nuclei and cellular debris. The postnuclear supernatant (PNS) concentration was determined by DC assay and adjusted to a final concentration of 1.5 mg/ml. Aliquotes (1.2 mg in 800 µl) of the PNS were pre-cleared for non-specific binding by adding 80 µl of protein A-coupled magnetic beads (Dynabeads, Invitrogen), previously washed three times with PBS 1x. Non-specifically bound material was eluted in 50 µl of *Laemmli buffer* 2x (1x: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% Bromophenol blue, 10% glycerol) and used a negative control. After pre-clearing, 3 µg of anti-Cav-1 (BD Transduction Laboratories) or 3 µg of normal rabbit IgG (as a negative control) added to the supernatant and the mixtures were stirred overnight at 4°C, thus allowing the immunocomplex formation. Immunoprecipitates were recovered by adding 80 µl of protein A-coupled magnetic beads, previously washed with PBS 1x, and by stirring the mixtures for 4 hours at 4°C. Beads were recovered by centrifugation at 1300 g for 5 minutes at 4°C, and the supernatant was collected as SNIP (supernatant after immunoprecipitation). Bound proteins were eluted in 50 µl of *Laemmli buffer* 2x and kept as IP (immunoprecipitate). The samples were analyzed by western blotting using specific antibodies. The conditions used for this immunoprecipitation experiment consent to recover not only the target protein, but also the proteins associated to the target allowing to determine which proteins interact with the targeted molecule.

Protein quantification

The protein quantification was performed through DC assay (Bio-rad). This colorimetric assay, similar to the Lowry assay, allows to quantify proteins in presence of different concentrations of detergents. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. In alkaline conditions, Cu^{2+} ions form coordination complexes with -NH groups of amino acids; these complexes interacting with the Folin reagent, determine its reduction. Amino acids, like tyrosine and tryptophan, bind the Folin reagent forming blue coloured complexes that can be detected by measuring the absorbance at 750 nm with the spectrophotometer.

The assay was performed in a 96 well plate following the protocol supplied with the Bio-rad DC assay kit. The samples diluted in *lysis buffer* (1% Triton X-100 (w/v), 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 milliunits/ml aprotinin) were analyzed in triple, like the protein standard, bovine serum albumin (BSA), at different concentrations. 25 µl of

reagent A and 200 µl of reagent B, both supplied with the kit, were added to each well. After 15 minutes of incubation, the absorbance at 750 nm was measured with the spectrophotometer. The samples reading were compared with the ones of the standard. The assay is linear between 1.5 and 7.5 µg/µl of protein amount.

Polyacrilamide gel electrophoresis

The samples were analyzed using electrophoresis on a polyacrilamide gel with denaturing conditions, a technique which allows to separate proteins previously denatured at a high temperature and in the presence of denaturing and reducing agents.
The samples were resuspended in *Laemmli buffer* (1x: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% Bromophenol blue, 10% glycerol) and boiled for 5 minutes at 100 °C before being analyzed. This buffer contains sodium dodecyl sulfate (SDS), an anionic detergent which binds most proteins in amounts roughly proportional to the molecular weight of the protein, about one molecule of SDS for every two amino acid residues. The bound SDS contributes a large net negative charge, rendering the intrinsic charge of the protein insignificant and conferring on each protein a similar charge-to-mass ratio. In addition, the native conformation of a protein is altered when SDS is bound, and most protein assume a similar shape. The presence of β-mercaptoethanol, which breaks disulfide bonds, and the high temperature also contribute to the loss of the native conformation of a protein. Electrophoresis in these conditions therefore separates proteins almost exclusively on the basis of the molecular weight, with smaller polypeptides migrating more rapidly.
The electrophoresis run was performed using a Miniprotean II unit, produced by Bio-rad. To obtain optimal resolution, a stacking gel is polymerized on top of the resolving gel. The stacking gel has a lower concentration of acrylamide, which determines larger pore size, lower pH and a different ionic content. This allows the proteins in a lane to be concentrated into a tight band before entering the running or resolving gel and produces a gel with tighter or better separated protein bands. After migrating through the stacking gel, the proteins enter the running gel where they separate on the basis of their molecular weight. A solution of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 was used as *running buffer*. The proteins were separated using 10% polyacrylamide gels.

Western blotting

After electrophoresis separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, at 200 mA for 3 hours at 4°C with a wet blotting (Mini Transblot Biorad). The transfer buffer used is *Blotting buffer* 1x (25 mM Tris-HCl, 192 mM glycine, 15 % methanol, pH 8.0-8.5). The membrane used for the protein transfer has a nonpolar nature therefore, before use, the PVDF membranes were wet in 100% methanol, to which increasing amounts of water were added until 100% of water and then equilibrated in blotting buffer 1x for at least 10 minutes. After the transfer, the PVDF membranes were immunoblotted using antibodies anti-caveolin-1 (BD Transduction Laboratories), anti c-Src (Cell Signaling), anti-integrin α5 (BD Transduction Laboratories), and anti-integrin β1 (BD Transduction Laboratories). Briefly, after the transfer the membrane was incubated in 5% milk defatted in TBS-T 0.05% (1 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween) to block the aspecific binding sites of the membrane. The membrane was then washed three times with TBS-T 0.05% and incubated with a specific antibody (primary antibody) either overnight at 4°C or 1 hour at room temperature, depending on the antibody used. The primary antibody was diluted in a solution of TBS-T 0.05% containing 1% bovine serum albumin (BSA). The membrane was washed again with TBS-T 0.05% for four times, to get rid of the antibody excess, before being incubated with the secondary antibody, a rabbit or mouse anti-IgG, depending on the origin of the primary antibody, conjugated with horseradish peroxidase (HPR) at room temperature for 45 minutes. The membrane was then washed again for six times and the peroxidase activity was assessed through incubation with a non-radioactive light emitting substrate for the detection of immobilized specific antigens conjugated with horseradish peroxidase-linked antibodies (LiteAbLot Plus, Euroclone) for 2 minutes. The luminescent compound generated following the reaction can be detected through exposition to a photographic film (Kodak BioMax MR Film, Sigma-Aldrich). The data acquisition was performed using a GS-700 Imaging Densitometer and acquired blots were elaborated using the Quantity One software (BioRad). Each experimental point was performed in triplicate, and data were expressed as the mean values ± S.D. of three independent experiments.

In some cases the membrane used for the Western Blotting underwent *stripping* to completely remove the protein-bounded antibodies and analyze the samples with other antibodies.

The membrane was incubated in a buffer containing Tris-HCl 62.5 mM, 2-mercaptoethanol 100 mM, 2% SDS pH 6.7 for 30 minutes at 50° C in agitation. The stripped membrane was then abundantly washed with TBS-T 0.05% until complete elimination of the *stripping buffer*. The membrane was then used in a new procedure of immunoblotting.

Table 2. Antibodies used for western blotting

Protein	Primary antibody			Secondary antibody (Incubation)
	Type	Blocking	Incubation	
Caveolin 1	Polyclonal, anti-rabbit	Overnight, 4°C	1 h, RT	45 min, RT
c-Src	Monoclonal, anti-rabbit	Overnight, 4°C	1 h, RT	45 min, RT
Integrin α5	Monoclonal, anti-mouse	2 h, RT	Overnight, 4°C	45 min, RT
Integrin β1	Monoclonal, anti-mouse	2 h, RT	Overnight, 4°C	45 min, RT

Lipid analysis

DRM fractions were prepared as described above. Lipids in PNS and in sucrose gradient fractions were extracted with chloroform/methanol/water 2:1:0.1 by volume (in the case of gradient fractions, water was omitted), and subjected to a two-phase partitioning, aqueous phase and organic phase. Then lipids in the aqueous phase or in organic phase were dried under N_2, and dissolved in the same volume of cholesterol/methanol, 2:1 by volume.

To determine the endogenous ganglioside content, the aqueous phase lipids were analyzed by HPTLC, using as solvent systems chloroform/methanol, 9:1 and chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 (spray reagent: Ehrlich) by volume, successively. In order to determine cholesterol, after methylation the organic phase lipids were analyzed by HPTLC, using as solvent systems Hexan:Ethyl acetate, 3:2 (spray reagent: Anisaldehyde) by volume. The endogenous phospholipid content in the organic phase was also analyzed by HPTLC, using as solvent systems

chloroform/Methanol/Acetic acid/H_2O, 30:20:2:1 (spray reagent: 15% H_2SO_4 in 1-Butanol) by volume. On HPTLC were loaded the same volume of each fraction. Data are expressed as nmol of lipids per mg protein.

ECM adhesion

Cell adhesion to fibronectin

Cell adhesion to defined matrix components was accomplished as previously described (230). In brief, flat-bottomed, polystyrene, 24-well plates were incubated overnight at 4 °C with 40 µg fibronectin in 250 µl of PBS (160 µg/ml) per well. Human fibronectin (Sigma) was used as substrates. Plates were washed with 500 µl of 1% bovine serum albumin (BSA) in PBS twice to remove unbound fibronectin and also to block any remaining reactive surfaces. Nonspecific cellular binding was determined by using wells coated only with 1% BSA. After the wells were washed with PBS, 1×10^5 cells per well in 250 µl of RPMI-1640 medium was plated, and the cells were incubated at 37 °C for 30 min for attachment to the fibronectin substrate. The cell adhesion was evaluated measuring the mitochondrial oxidative activity through MTT reduction assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), yellow and soluble, is incorporated from the cell and reduced by the mitochondrial succinate dehydrogenase with the formation of purple formazan crystals, insoluble in aqueous solution. After nonadherent cells were washed off, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to the culture and incubated at 37 °C for 3 h, to allow the compound reduction, and then 250 µl of soluble solution (0.1 M HCl in 10% SDS (w/v) solution) was added and mixed completely, and then was incubated at 37 °C overnight, to dissolve the formazan crystals. Optical density (absorbance at 570 nm minus that at 650 nm) was measured to evaluate cells attached to the substrate.

ECM adhesion assay

Cell adhesion to different extracellular matrix proteins was assessed using an ECM Cell Adhesion Array Kit (ECM540, Millipore), which utilizes a colorimetric detection format. The kit contains a 96-well plate consisting of 12 × 8-well removable strips. Seven wells of each strip are pre-coated with a different ECM protein (Collagen type I, collagen type II, collagen type IV, fibronectin, laminin, vitronectin, and tenascin), while

one well is coated with bovine serum albumin (BSA). The BSA-coated well is used to determine non-specific cell adhesion. The cell adhesion was tested in different conditions (wild type cells and 4T untreated cells; three replicates for each condition) following the protocol supplied with the adhesion kit.

A2780 wild type and A2780/SAT-I 4T cells were cultured in 60 mm Petri dishes. The cells were collected and counted, and the cell suspension was centrifugated at 1100 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in the assay buffer supplied with the kit. 1.6 x 10^4 cells were added to each well of the ECM array plate and incubated for 10 minutes at 37 °C with 5% CO_2. After incubation, the wells were washed thrice with assay buffer before adding 100 µl of cell stain solution and incubating the plate for 5 minutes at room temperature. The stain was then removed and the wells were washed with deionized water for 5 times. The wells were allowed to dry for a few minutes, then 100 µl of extraction buffer, a solution containing alcohol, were added to each well. The strips were incubated at room temperature on an orbital shaker until the cell-bound stain was completely solubilized (about 10 minutes). Absorbance at 570 nm was measured with a spectrophotometer.

Determination of the linearity range of the crystal violet dye

The cell stain solution used for the ECM adhesion assay, supplied with the ECM Cell Adhesion Array Kit (ECM540, Millipore), contains crystal violet, a dye that colors intact living cells. To verify the linearity range of the dye, A2780/SAT-I 4T monoclonal cells were grown in 60 mm diameter Petri dishes until they reached 90 % of confluence. The cells were collected, counted and resupended in array buffer. Different amounts of cells were then seeded at different concentrations (0, 100, 1 x 10^4 - 3 x 10^4 cells/well; three replicates for each concentration) on a 96-well plate and incubated at 37 °C, 5% CO_2 for 2 hours. The wells were then washed three times with the assay buffer and 100 µl of cell stain solution were added to each well. After 5 minutes of incubation at room temperature, the stain was removed and the wells were washed with deionized water for 5 times. The wells were allowed to dry for a few minutes, then 100 µl of extraction buffer were added to each well. The strips were incubated at room temperature on an orbital shaker until the cell bound stain was completely solubilized (about 10 minutes). Absorbance at 570 nm was measured with a spectrophotometer.

siRNA transfection

A2780/HPR or A2780/SAT-I cells were plated in 6-well plates or 100-mm dishes and, when grown at 50% confluence, were transfected with CAV1 siRNA (Qiagen, cat. no. SI00299635) or with scrambled siRNA duplexes (Qiagen, All stars negative control siRNA cat. no. 1027280) as transfection control. The optimal condition for the transfection was 32 nM siRNA in Opti-MEM with Lipofectamine 2000 (1%, v/v) (Invitrogen), following the protocol provided by the manufacturer. Fresh medium was added 24 h after transfection, and experiments were conducted for different times up to 72 h. In the case of the cell adhesion assay, cells were pre-treated with siRNA for 48 h before the assay.

Statistical analysis

Experiments were run in triplicate, unless otherwise stated. Data are expressed as mean value ± S.D. and were analyzed by oneway analysis of variance followed by Student-Neuman-Keul's test. *p*-values are indicated in the legend of each figure.

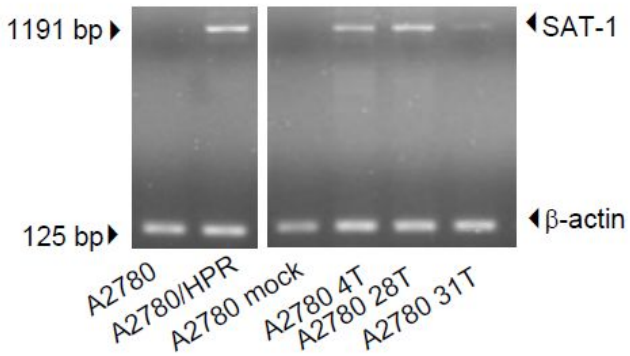
RESULTS

GM3 synthase overexpression in A2780 cells

In order to investigate the role of gangliosides in the regulation of tumor phenotype , we stably overexpressed the cDNA enconding sialyltransferase-I (GM3 synthase or SAT-I) in A2780 human ovarian carcinoma cells. The sialyltransferase-I is a key enzyme which controls the sialylation step leading to the synthesis of ganglioside GM3 from lactosylceramide (LacCer).

As shown in Figure 8, the SAT-I mRNA levels (Panel A) were markedly up-regulated in the three SAT-I transfected clones (4T, 28T and 31T) with respect to wild type and mock transfected A2780 cells. Consistently, cellular ganglioside content was higher in SAT-I tranfectants (Figure 8, Panel B). The increase in the gangliosides content in SAT-I transfectants was due to higher levels of GM3, GM2, and GD1a. The mRNA level of GM3 synthase and gangliosides content in fenretinide-resistant A2780/HPR cells were also detected. Both of the mRNA level of SAT-I and gangliosides content were much higher compared to A2780 wild type cell.

A



B

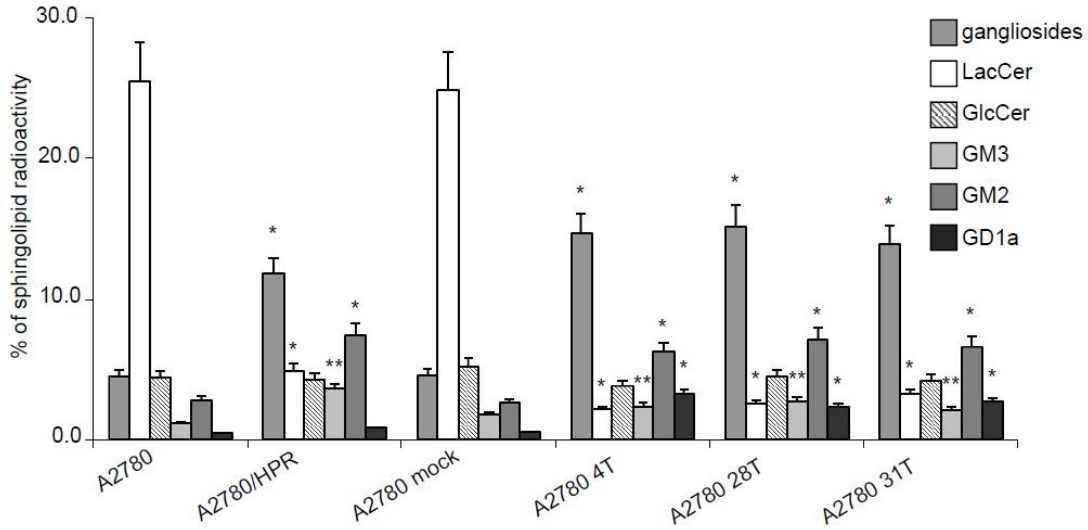


Figure 8. SAT-I expression and glycosphingolipid composition in A2780 variants. ***Panel A***, SAT-I mRNA levels were assessed in wild type A2780 cells, A2780 transfectants (one mock and 3 SAT-I-transfected clones, 4T, 28T, and 31T) and A2780/HPR cells by reverse transcriptase PCR. β-Actin mRNA expression was measured as an internal control. Patterns are representative of those obtained in three different experiments. ***Panel B***, cell lipids were metabolically labeled with [1-^{3}H]sphingosine as described in *Materials and methods*. Lipids were extracted with chloroform/methanol/water. The total lipid extracts and the partitioned aqueous and organic phase lipids were separated by HPTLC. Radioactive lipids were visualized by digital autoradiography. The radioactivity associated with each lipid was determined with specific Beta-Vision software. Data are expressed as percentage of the total radioactivity incorporated into sphingolipids and are the means ± S.D. of three different experiments. $^{*}P < 0.01$ versus A2780. $^{**}P < 0.05$ versus A2780.

Elevated cellular ganglioside levels reduced *in vitro* motility of A2780 cells

We investigated the effect of increased GM3 synthase expression levels on A2780 *in vitro* motility (Figure 9,A). Overexpression of GM3 synthase did not significantly affect the growth rate of A2780 cells (data not shown). On the other hand, wound healing assay revealed that the *in vitro* motility of all SAT-I transfectants (4T, 28T and 31T) was strongly reduced when compared with wild type or mock-transfected A2780 cells. A2780/HPR cells which endogenously express high levels of GM3 synthase, also showed a lower *in vitro* motility than A2780 cells. Above all, high levels of GM3 synthase expression were sufficient to negatively regulate the *in vitro* motility of A2780 human ovarian carcinoma cells without influencing the cell growth and this may be due to the consequent increase of cellular ganglioside levels.

To validate and substantiate the hypothesis that changes in the ganglioside patterns consequent to SAT-I overexpression could be responsible for the reduced motility in A2780 cells, we incubated A2780 cells in the presence of exogenous gangliosides under experimental conditions that allowed us to increase by 3- to 20-fold the trypsin-stable cellular content of the administered gangliosides (data not shown). Under these

conditions, we observed that the exogenous administration of GM3 and GM2, both natural components of A2780 cells, and of GM1, virtually absent from these cells, effectively reduced the *in vitro* motility of these cells (Figure 9,B). On the other hand, GD1a, present as minor ganglioside in A2780 cells but representing 15% - 22% of total gangliosides of SAT-I-transfected clones, had no effect on A2780 cell motility.

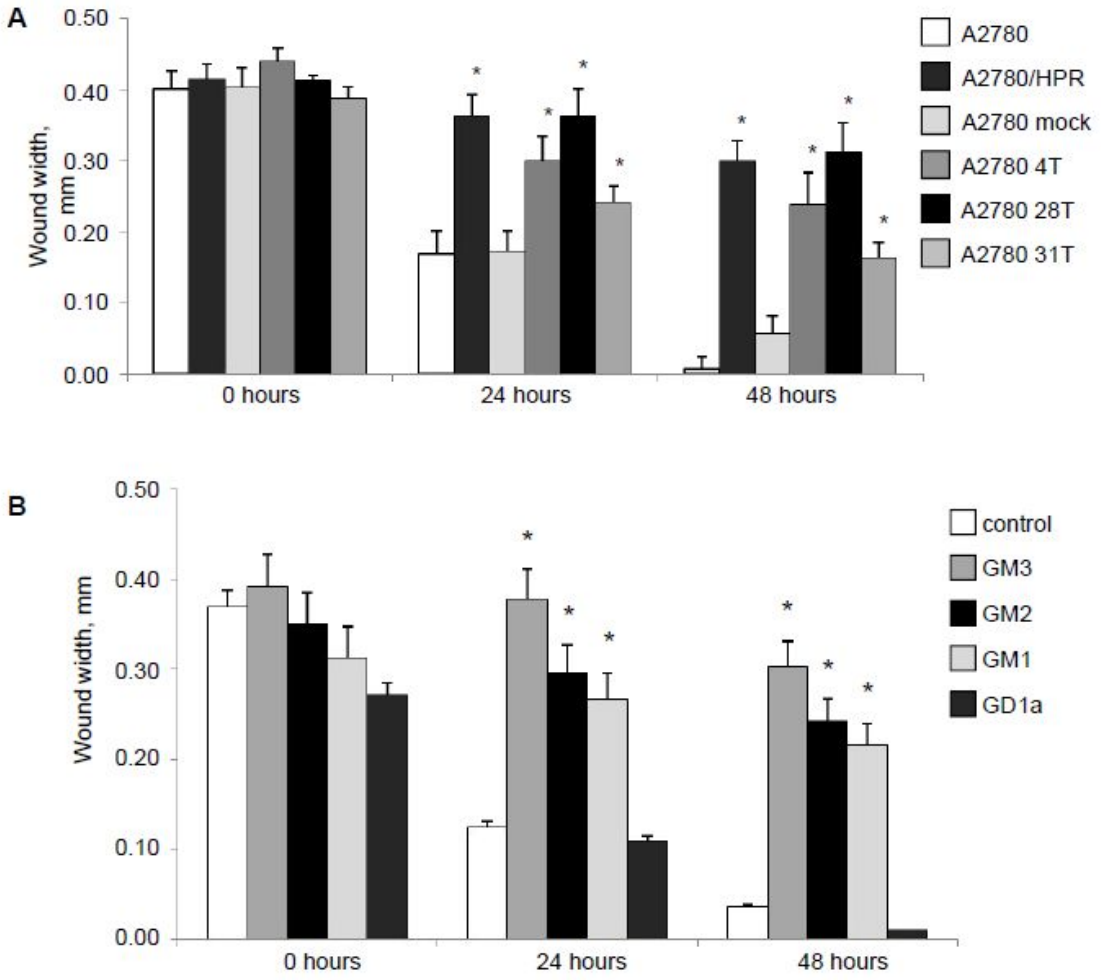


Figure 9. Effect of elevated SAT-I expression and exogenous administration of gangliosides on *in vitro* motility of A2780 cells. ***Panel A***, *in vitro* motility of A2780 cells, A2780 transfected with the empty expression vector (mock) or the vector containing the SAT-1 cDNA (clones 4T, 28T and 31T), and A2780/HPR cells has been assessed by a wound healing assay as described in *Materials and Methods*. Confluent monolayers were wounded with a rubber policeman, the wound width has been measured at 0, 24 and 48 hours. Data are expressed in mm, and are the means ± S.D. of three different experiments. *, $P < 0.01$ versus time-

matched A2780. ***Panel B***, A2780 confluent monolayers were wounded and incubated in serum-free medium in the presence of vehicle(control) or of 50 μM GM3, GM2, GM1 or GD1a for up to 48 hours. The wound width has been measured at 0, 24 and 48 hours. Data are expressed in mm, and are the means ± S.D. of three different experiments. *, $P < 0.01$ versus time-matched control.

Elevated cellular ganglioside levels increased *in vitro* adhesion of A2780 cells to fibronectin

The α5β1 integrin, which is the most expressed integrin heterodimer in A2780 cells, has been reported to bind to the extracellular matrix components fibronectin, type I collagen, and laminin (231). The ability of the SAT-I transfected monoclonal cells to adhere to fibronectin, a major ligand of α5β1 integrin, was compared with that of A2780 wild-type cells and A2780/HPR cells (Figure 10,A). Adhesion to bovine serum albumin (BSA) was also examined as a non α5β1 integrin-mediated adhesion control. Adhesion was examined after a 30 minutes incubation of the cells on the coated wells using a MTT reduction assay as described in the *Materials and Methods*. After 30 minutes of incubation, there was nearly no cell binding to BSA coated wells. On the fibronectin coated wells, both SAT-I transfected cells and A2780/HPR cells had a strong adhesive ability compared to A2780 wild-type cells. Since the cell adhesive ability usually has a reversed behavior of *in vitro* cell motility, this data is consistent with the motility results observed above. GM3 synthase overexpression resulted in an increase of cell adhesion, and also in a decrease of *in vitro* cell motility, suggesting a possible role of gangliosides in controlling integrin-mediated cell motility and adhesion.

To further verify if the content of gangliosides was related to the cell adhesion, exogenous gangliosides were administered to A2780 cells. Briefly, A2780 cells were pre-treated with gangliosides for 48 hours as described in the *Materials and Methods*. The cell adhesion assay was conducted by using fibronectin-coated 24-well plates and adhesion was measured using an MTT reduction assay as described in *Materials and Methods*. As shown in figure 10, there was no significant difference of the tendency of binding to BSA-coated wells among control, gangliosides GM3- and GD1a-treated A2780 cells (Panel B). However the cells treated with gangliosides GM3, attached more strongly to the fibronectin-coated wells, compared to control and to cells treated with

GD1a. Since exogenous administration of ganglioside GM3 significantly reduced the A2780 cell motility, but GD1a did not, we supposed that in A2780 human ovarian carcinoma cells, high levels of ganglioside GM3 resulted in a relatively high adhesion, and thus in reduced *in vitro* cell motility. Notably, fibronectin, as an important extracellular matrix protein, has a specific affinity for integrin α5β1, which is the main type of integrin expressed in A2780 cells, suggesting that integrin α5β1 is involved in GM3-mediated regulation of *in vitro* motility and adhesion in A2780 cell.

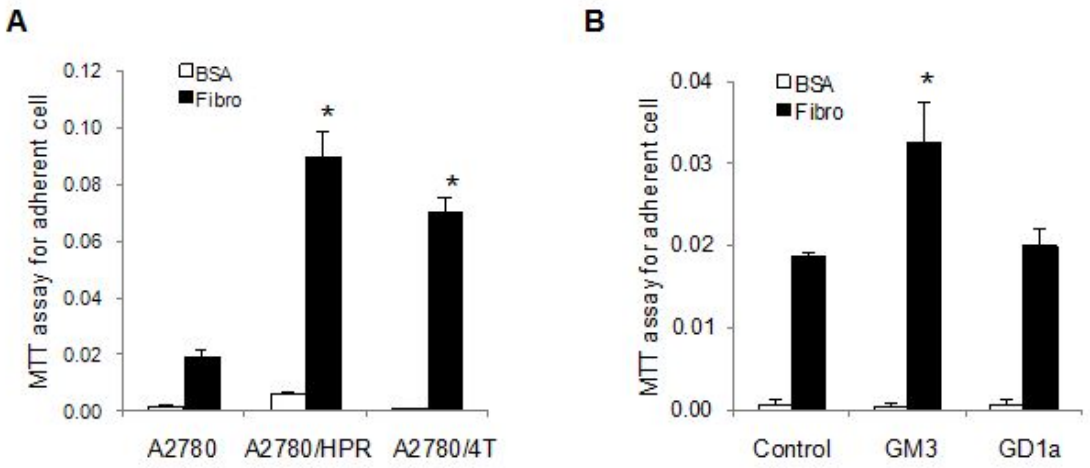


Figure 10. Effect of elevated SAT-I expression and exogenous administration of gangliosides on *in vitro* adihesion of A2780 cells. Human fibronectin and bovine serum albumin (BSA) were coated in 24-well plate at 4 °C overnight. On the other day, the plate was washed with PBS before use. ***Panel A***, 1×10^5 of A2780, A2780/HPR and A2780/SAT-I cells were respectively plated in each well and incubated at 37°C for 30 min allowing cell adhesion. Then non-adherent cells were washed off. MTT assay was performed to evaluated the number of adherent cells as described in *Materials and methods*. Data are the means ± S.D. of three different experiments. *, $P < 0.05$ versus A2780 cells. ***Panel B***, A2780 cell confluent monolayers were pre-treated with vehicle (control) or 50 μM GM3 or GD1a in serum-free culture medium for 48 hours. After that, cell adhesion was evaluated following the procedure as described in *Materials and methods*. Data are the means ± S.D. of three different experiments. *, $P < 0.05$ versus control, cells treated with vehicle only.

Gangliosides depletion reduced *in vitro* motility and increased adhesion of A2780/SAT-I cells

High expression levels of GM3 synthase, leading to high cellular gangliosides content, in A2780/SAT-I-transfected cells were associated with a reduction of *in vitro* cell motility and an increased cell adhesion. Consistently, treatment with exogenous gangliosides was able to reduce the motility and increase the adhesion of low GM3 synthase A2780 cells, suggesting a role of gangliosides in controlling the motility and adhesion of these cells. To conform this hypothesis, we assessed the effect of the pharmacological manipulation of ganglioside levels on the *in vitro* motility and adhesion of A2780/SAT-I cells. Treatment of A2780/SAT-I cells with the specific GlcCer synthase inhibitor D-PDMP strongly reduced gangliosides content after 2 days (Figure 11,A). L-PDMP (the ineffective isomer, used as a negative control) had no effect on glycosphingolipid levels in A2780/SAT-I cells, as expected. Both of L- and D-PDMP did not alter the cellular levels of ceramide and sphingomyelin (SM) in A2780/SAT-I cells. Thus, D-PDMP treatment can be an appropriate tool to study the role of gangliosides in A2780 cells.

Treatment with L- or D-PDMP was not toxic; however, it slightly reduced A2780/SAT-I cell proliferation in a dose-dependent manner (data not shown). Since PDMP treatment influenced cell number, phagokinetic gold sol assay (PGSA) was carried out to determine *in vitro* cell motility instead of wound healing assay. Phagokinetic gold sol assay is highly independent from the cell number and analyzes the migration behavior of the single cell. The assay is based on the principle that migrating cells ingest, push to one side, or collect small particles on their dorsal surface in their path. At least fifty of single cells were analyzed for each situation. Treatment with D-PDMP, but not with L-PDMP, was able to significantly increase the motility of A2780/SAT-I cells (Figure 11,B). Above all, these results further suggest a role for gangliosides in the regulation of A2780 human ovarian cancer cell motility, demonstrating that the high content of gangliosides resulted in low cell *in vitro* motility; while on the contrary, low content of gangliosides resulted in high cell motility.

Since cell motility is tightly correlated with the adhesive ability, we supposed that PDMP treatment may also affect cell adhesion. To confirm this hypothesis, we assessed the effect of the pharmacological manipulation of ganglioside levels on the *in vitro* adhesion of A2780/SAT-I transfected cells. A2780/SAT-I 4T monoclonal cells were pretreated with PDMP for 48 hours to inhibit the endogenous gangliosides synthesis. The treatment with L- or D-PDMP did not shown toxicity at the concentration used for this experiment. The adhesive ability of these cells was analyzed following the cell

adhesion assay protocol as described in *Materials and Methods*. D-PDMP treatment significantly reduced the cell adhesion to fibronectin, while treatment with L-PDMP, the inactive stereoisomer used as a negative control, had no effect on cell adhesion, which was similar to the untreated control (Figure 11,C). The correlation between high GM3 content and decreased motility/increased adhesion, and the results obtained upon ganglioside depletion, indicate that gangliosides, especially GM3, might play an important role in modulating both cell motility and adhesion in A2780 cells. The mechanism of this regulation and if other molecules also take part in this procedure though need to be further investigated.

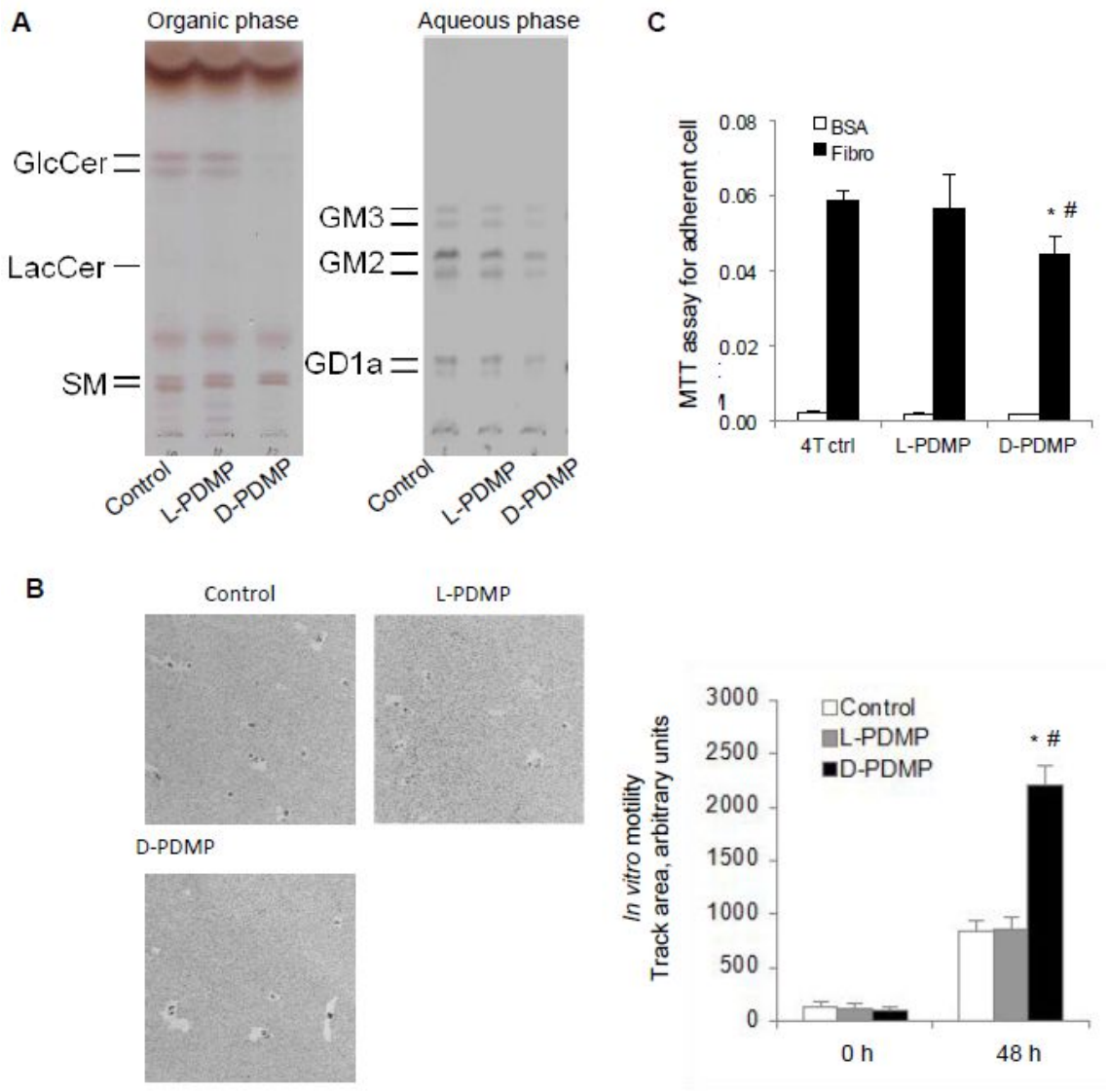


Figure 11. Effects of PDMP treatment on sphingolipids composition and on *in vitro* cell motility and adhesion of A2780/SAT-I cells. A2780/SAT-I 4T cells were treated with the specific glucosylceramide

synthase inhibitor D-PDMP to achieve sphingolipid depletion. The ineffective stereoisomer L-PDMP has been used as a negative control. ***Panel A***, Sphingolipid patterns of A2780/SAT-I cells untreated (control), treated with 20μM L-PDMP or D-PDMP for 48 hours. Cell lipids were extracted with chloroform/methanol/water, 2:1:0.1 by volume, subjected to a two-phase partitioning. Organic phase (left panel) and Aqueous phase (right panel) lipids were analyzed by HPTLC, using chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 by volume (spray reagent, p-dimethylaminobenzaldehyde) for aqueous phases; and chloroform/methanol/water, 55:20:3 by volume (spray reagent, 15% H_2SO_4 in 1-butanol) for organic phases as solvent system respectively. The equivalent to 1 mg of cell proteins were loaded on each lane. ***Panel B***, effect of PDMP on *in vitro* cell motility of A2780/SAT cells. A2780/SAT-I 4T cells were treated with 10 μM of L- or D-PDMP for 48 hours. Phagokinetic gold sol assay (PGSA) was performed as described in *Materials and Methods*. The L- or D-PDMP was matained in the medium for the whole duration of the assay. Phase-contrast microscopy images of the areas of the tracks cleared by the cells (left panel shows representative images for each data set) have been recorded at time 0 and after 48 hours. Average track areas (means ±S.D. of 50 measurements) normalized for the different cell size are reported in the bar graph (right panel). ***Panel C***, effects of PDMP treatment on *in vitro* adhesion of A2780/SAT-I cells has been assessed by cell adhesion assay as described in *Materials and methods*. Data are the means ± S.D. of three different experiments. *, $P < 0.05$ versus control. $^{\#}$, $P < 0.05$ versus L-PDMP-treated cells.

In vitro cell adhesion to ECM proteins of A2780 and A2780/SAT-I cells

The principal receptors on animal cells for binding most extracellular matrix proteins are the integrins, a family of heterodimeric transmembrane receptors consisting of noncovalently bound α and β subunits. Most integrins recognize several ECM proteins. Conversely, individual matrix proteins, such as fibronectin, laminins, collagens, and vitronectin, bind to several integrins. For example at least eight types of integrin bind fibronectin, and at least five types bind laminin. In A2780 cells, α5β1 integrin is the

main type of integrin expressed and it has been hypothesized that this integrin heterodimer might play a role in the regulation of GM3-mediated adhesion and motility. However α5β1 integrin is not the only integrin receptor expressed by A2780 cells, therefore other heterodimers might be involved in the regulation of adhesion and motility. To verify if other integrin heterodimers could be involved in such processes, we assessed the adhesive ability of A2780 cells to different extracellular matrix proteins. To do so we performed an adhesion assay using an ECM cell adhesion array kit (Millipore). This kit allows to test adhesion to 7 different ECM proteins, namely collagen I, II, and IV, fibronectin, laminin, vitronectin, and tenascin. Bovine serum albumin (BSA) was used to determine non-specific cell adhesion. The assay was performed on A2780 wild type and A2780/SAT-I 4T cells as described in *Materials and Methods*. Briefly, cells were collected, counted and seeded on the pre-coated wells. After 10 minutes of incubation at 37 °C with 5% CO_2, adhesion was measured using a colorimetric assay. The cells were stained with a solution containing crystal violet, which colors living cells, and, after washing the wells with deionized water, the cells were incubated for 10 minutes at room temperature with an extraction solution containing alcohol. Optical density was measured at 570 nm with a spectrophotometer. The linearity range of the staining solution was verified by seeding different concentration of cells on an uncoated 96-well plate and using the same colorimetric detection method as described in *Materials and Methods* (data not shown).

As shown in figure 12, the two groups of cells showed a very weak adhesion to collagen type I, II, and IV, and to tenascin suggesting that the integrin heterodimers involved in the recognition of these proteins might be expressed only at a low level or not expressed at all. On the other hand, the two groups of cells showed a slightly stronger adhesion to laminin and vitronectin and an even higher adhesion to fibronectin. While the latter was expected, since fibronectin has a specific affinity for α5β1 integrin and this heteodimer is the most expressed in A2780 cells, the adhesion to laminin and vitronectin suggests that integrin heterodimers such as αVβ1 and αVβ5, which bind vitronectin, or α3β1 and α6β1, which bind laminin, might be involved in regulating the adhesion of A2780 cells.

The adhesion assay also allowed us to compare A2780 wild type cells and A2780/SAT-I 4T cells. As expected, since it has been previously observed that cells with higher ganglioside content also have higher adhesion, 4T cells showed a higher adhesion to fibronectin and laminin. The involvement of integrin α5β1 in regulating the adhesion, and motility, in A2780 wild type and A2780/SAT-I cells has been further investigated

by analyzing the effect of PDMP treatment on this subunits distribution in gradient fractions and by investigating the effect of the same treatment on the association between these integrin subunits and other proteins, such as caveolin-1 and c-Src, involved in the formation of a molecular complex involved in the regulation of cellular processes.

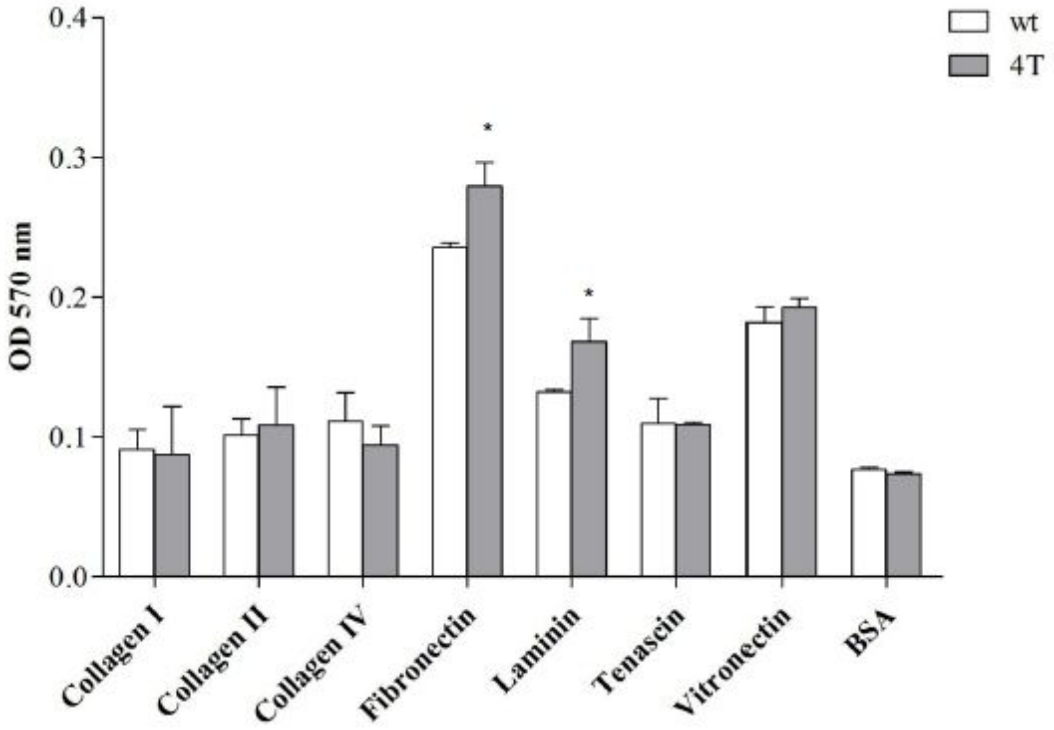


Figure 12. *In vitro* cell adhesion to ECM proteins. A2780 wild type cells were compared to A2780/SAT-I cells. The cell adhesion to collagen I, II, and IV, fibronectin, laminin, tenascin and vitronectin was assessed using an ECM cell adhesion array kit following the protocol described in *Materials and Methods*.

Effects of PDMP treatment on lipids gradient distribution in A2780/SAT-I cells

To address the mechanism underlying regulation of cell motility and adhesion by gangliosides, the effect of ganglioside depletion by PDMP treatment on the association of cellular components with detergent-resistant membrane fraction has been evaluated. Firstly, the mass content for each lipid or lipid class has been determined

colorimetrically as described in *Materials and methods*. As shown in Table I, lipid levels were not affected by L-PDMP treatment. However, D-PDMP treatment significantly reduced ganglioside content of A2780/SAT-I, leaving unaffected the total phospholipids and cholesterol levels.

	Total phospholipids	Cholesterol	Gangliosides
Control	193.7 ± 29.6	47.3 ± 5.7	2.87 ± 0.23
L-PDMP	165.9 ± 13.6	53.8 ± 3.1	2.35 ± 0.26
D-PDMP	174.7 ± 20.0	48.2 ± 6.9	0.79 ± 0.08*#

Table 1. **Effect of PDMP treatment on the endogenous lipids content in A2780 SAT-I-transfected clones**. The mass content for each lipid or lipid class has been determined colorimetrically as described in *Materials and methods*. Data are expressed as nmol of lipid per mg of cell protein and are the mean values ± S.D. of three different experiments. *, $P < 0.05$ versus control. $^{\#}$, $P < 0.05$ versus L-PDMP-treated cells.

In order to analyze the effect of ganglioside depletion on the membrane organization in these cells, we prepared a ganglioside- and caveolin-1 enriched detergent resistant membrane (DRM) fraction from SAT-I transfected cells treated with L- or D-PDMP. To do this, cells were lysed in the presence of Triton X-100 and subjected to discontinuous sucrose gradient centrifugation as described in *Materials and methods*. As expected, the low density detergent resistant membrane fraction (fractions 4, 5 and 6) was highly enriched in gangliosides. Gangliosides levels were significantly reduced in all fractions in D-PDMP treated cells (Figure 13,A). In addition, D-PDMP treatment determined a significant shift of cholesterol from both DRM fraction and intermediate fraction (fractions 7 and 8) to high density fraction (Figure 13,B). This movement may be caused by the dynamic of caveolin-1 (Figure 14,A); however, the mechanism is needed to be explored. Glycerophospholipids content of each fraction was also analyzed by HPTLC. The major amount of glycerophospholipids existed in high density fraction (fractions 9, 10 and 11). Glycerophospholipids level were reduced in DRM fraction and intermediate fraction, and increased in high density fraction by D-PDMP treatment in this case (Figure 13,C).

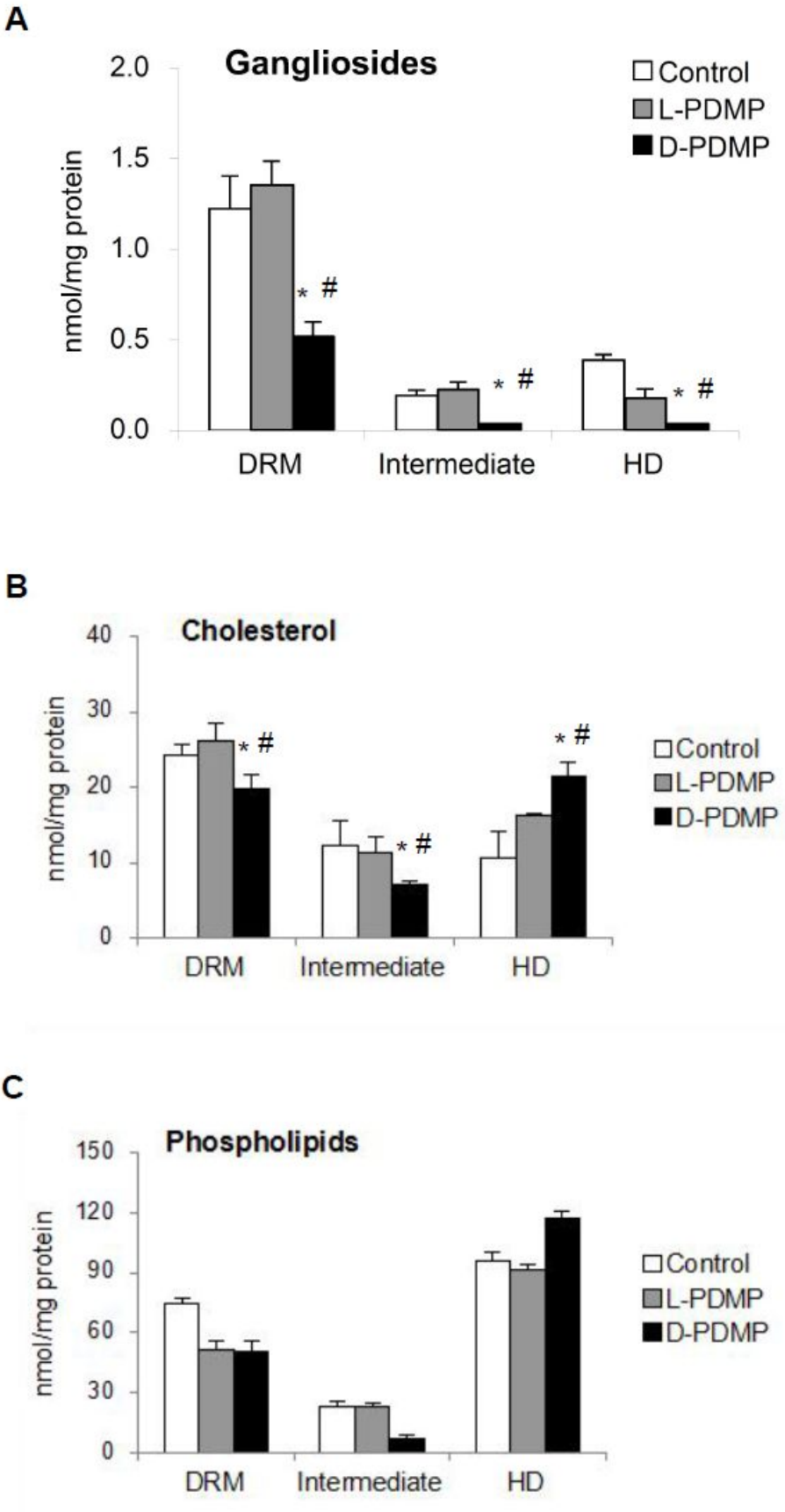

A
Gangliosides
Control
L-PDMP
D-PDMP
nmol/mg protein
2.0
1.5
1.0
0.5
0.0
* #
DRM
Intermediate
HD
B
Cholesterol
nmol/mg protein
40
30
20
10
0
Control
L-PDMP
D-PDMP
DRM
Intermediate
HD
C
Phospholipids
nmol/mg protein
150
120
90
60
30
0
Control
L-PDMP
D-PDMP
DRM
Intermediate
HD

Figure 13. Effects of PDMP treatment on lipids distribution in A2780/SAT-I cells. A2780/SAT-I 4T cells were treated with or without L-/D-PDMP for 48 hours. The low-density fraction 4, 5 and 6 were pooled together to obtain the DRM fraction, similarly the fraction 7 and 8 were pooled together to obtain the intermediate fraction, whereas fractions 9, 10 and 11 were pooled together to obtain the high density (HD) fraction. Lipids present in the pooled fractions were extracted and analyzed as described under *Materials and methods*. ***Panel A***, effect of PDMP treatment on gangliosides distribution in A2780/SAT-I transfected clones. ***Panel B***, effect of PDMP treatment on cholesterol distribution in A2780 SAT-I-transfected clones. ***Panel C***, Effect of PDMP treatment on phospholipids (PC and PE) distribution in A2780/SAT-I transfected clones. Data are expressed as nmol of lipid per mg of cell protein and are the means ± S.D. of three different experiments. *, $P < 0.05$ versus control. $^{\#}$, $P < 0.05$ versus L-PDMP-treated cells.

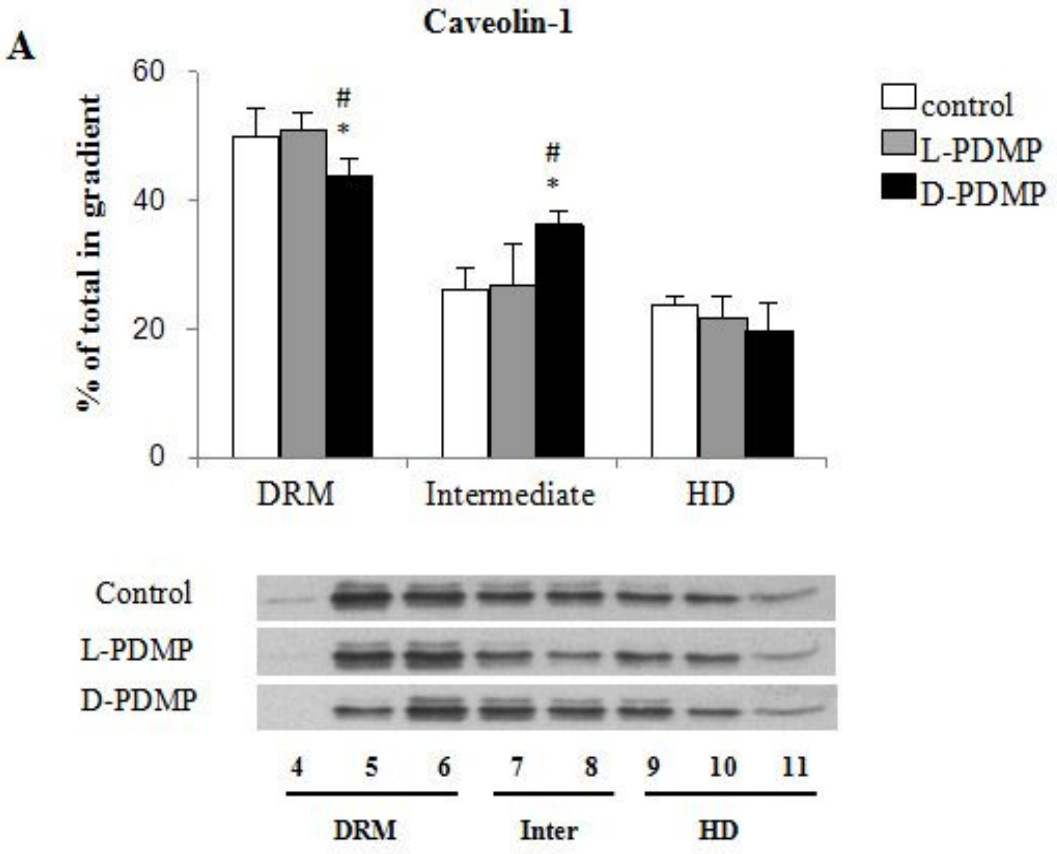
Effects of PDMP treatment on protein gradient distribution in A2780/SAT-I cells

Glycosphingolipids clusters at the cell surface membrane interact with functional membrane proteins such as integrins, growth factor receptors, tetraspanins, and non-receptor cytoplasmic protein kinases to form "glycosynaptic domains" controlling cell growth, adhesion, and motility. Data previously obtained (5) suggests a role for gangliosides in regulating tumor cell motility by affecting the function of a signaling complex organized by caveolin-1, responsible for Src inactivation downstream to integrin receptors, and imply that GM3 synthase is a key target for the regulation of cell motility in human ovarian cancer. To analyze the effects of PDMP treatment on proteins distribution, we prepared a ganglioside- and caveolin-1 enriched detergent resistant membrane (DRM) fraction from SAT-I transfected cells treated with L- or D-PDMP under the same experimental conditions as described above.

The distribution of caveolin-1, c-Src, integrin α5 and β1 along the gradient fractions was analyzed by immunoblotting using specific antibodies. The DRM fraction, which is enriched in sphingolipids (ceramide, GlcCer, sphingomyelin, and gangliosides), was also highly enriched in caveolin-1, whereas integrin α5 was largely recovered in the

high density fraction of the gradient, which also contains the major amount of glycerophospholipids .

Treatment with D-PDMP, but not L-PDMP, was able to induce the shift of a significant amount of caveolin-1 to intermediate fraction from both DRM fraction and high density fraction (Figure 14,A) and the shift of a significant quantity of c-Src from the DRM fraction to the intermediate fraction (Figure 14,B). In control cells, the amount of integrin α5 and β1 associated with the DRM fraction was very low. After D-PDMP treatment, integrin α5 moved from the high density fraction, to the DRM fraction and intermediate fraction (Figure 15,B). D-PDMP treatment also determined a shift of integrin β1 from the HD fraction to the intermediate fraction (Figure 15,A). Although the distributions of caveolin-1, c-Src and integrin α5 were altered, the total expressions of these proteins were not changed by PDMP treatment,.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60
40
20
0
#
*
#
*
control
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DRM
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HD
Control
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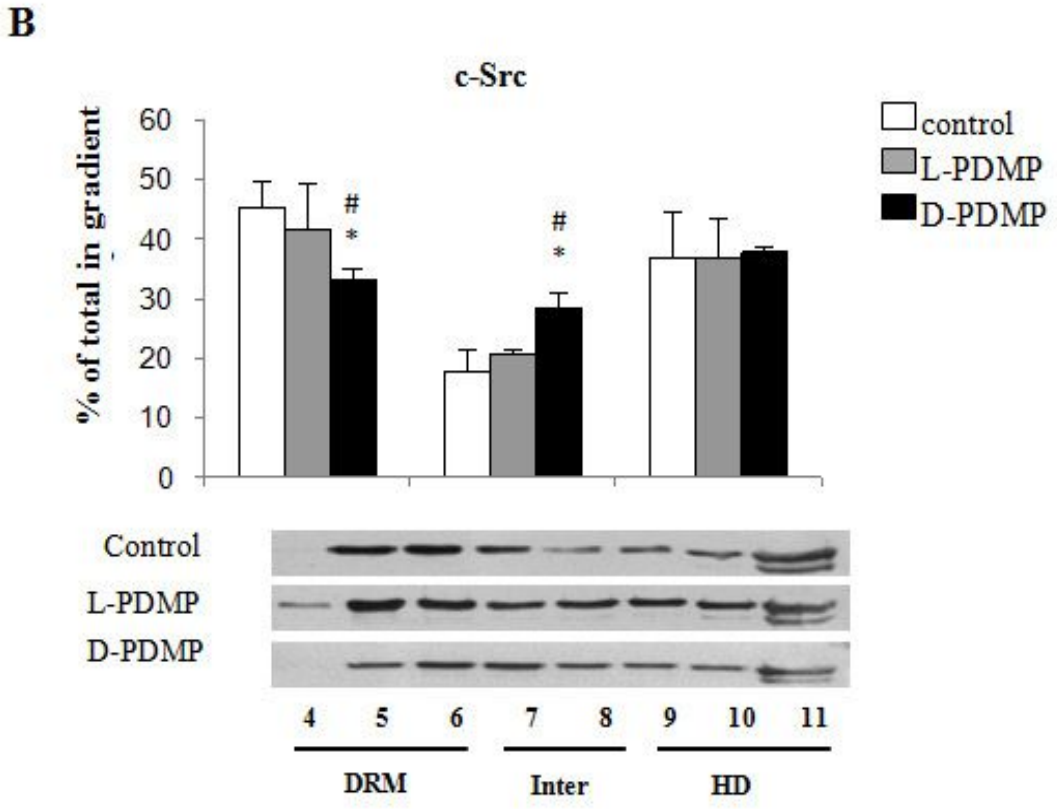
D-PDMP
4 5 6 7 8 9 10 11
DRM
Inter
HD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c-Src
% of total in gradient
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50
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10
0
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control
L-PDMP
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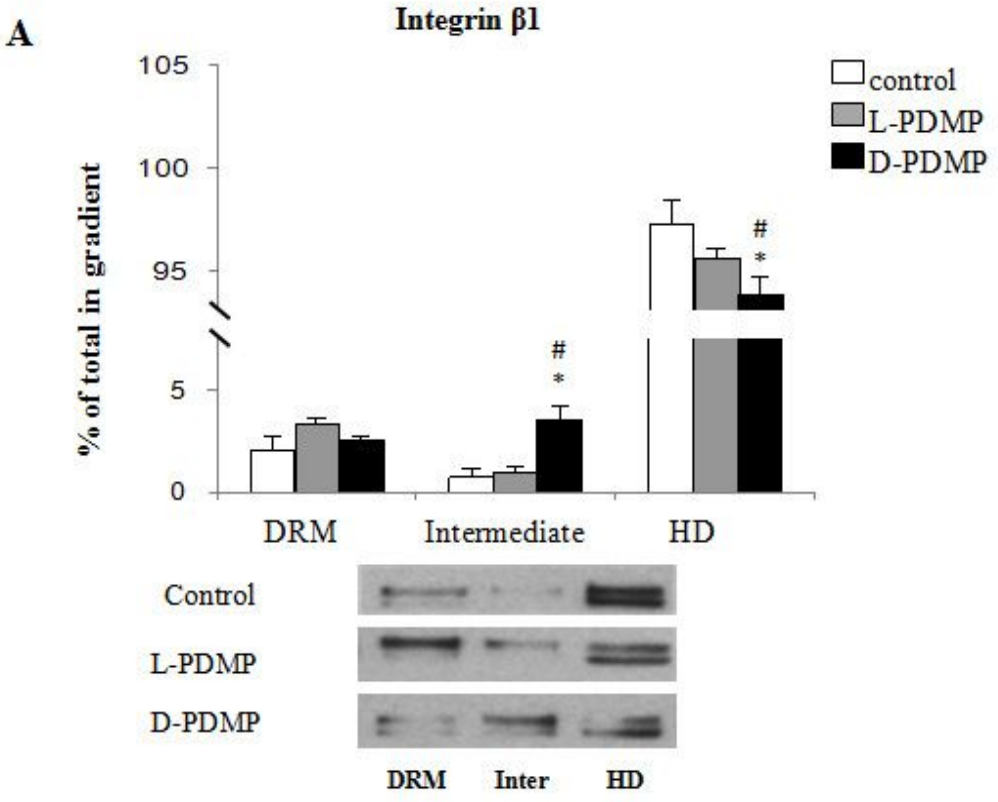
Control
L-PDMP
D-PDMP
4 5 6 7 8 9 10 11
DRM
Inter
HD

Figure 14. Effects of PDMP treatment on protein gradient distribution in A2780/SAT-I cells. Cells were treated with 20 μM L- or D-PDMP for 48 hours. Cell gradient fractions were prepared by sucrose gradient centrifugation after lysis in the presence of 1% Triton X-100 as described under *Materials and methods*. The protein distribution was determined by SDS-PAGE followed by Western blotting, using specific antibodies against caveolin-1 (Panel A) and c-Src (Panel B). The relative quantities of each protein in pooled DRM (fractions 4-6), Intermediate (fractions 7-8) and HD (fractions 9-11) fractions were calculated by densitometry and were expressed as percentage of total signal assessed in the histogram. The loading volume is 1/100 of the total volume of each fraction. Patterns in the lower part of each panel are representative of those obtained in three independent experiments. *, $p < 0.05$ *versus* control. $^{\#}$, $p < 0.05$ *versus* L-PDMP treated cells.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Integrin β1
105
100
95
5
0
% of total in gradient
control
L-PDMP
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Intermediate
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Control
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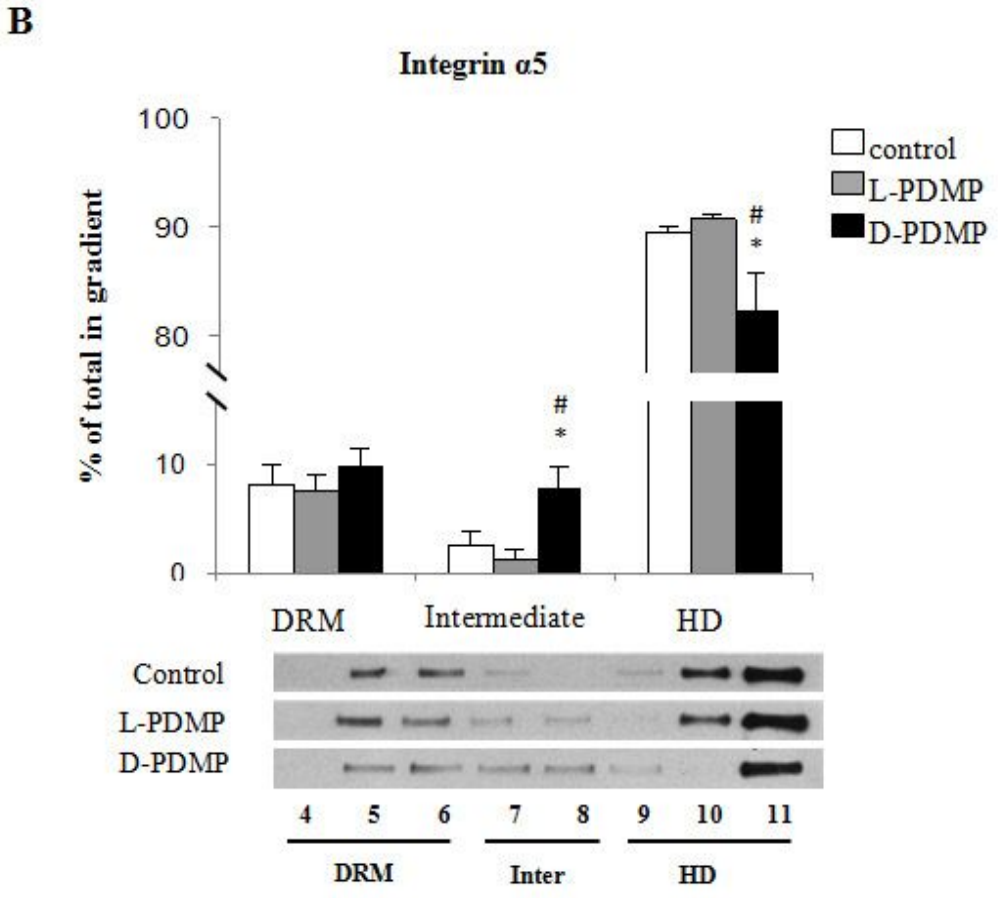
HD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100
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DRM
Intermediate
HD
Control
L-PDMP
D-PDMP
4
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6
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8
9
10
11
DRM
Inter
HD

Figure 15. Effects of PDMP treatment on protein gradient distribution in A2780/SAT-I cells. Cells were treated with 20 μM L- or D-PDMP for 48 hours. Cell gradient fractions were prepared by sucrose gradient centrifugation after lysis in the presence of 1% Triton X-100 as described under *Materials and methods*. The protein distribution was determined by SDS-PAGE followed by Western blotting, using specific antibodies against integrin α5 (Panel A) and β1 (Panel B). The relative quantities of each protein in pooled DRM (fractions 4-6), Intermediate (fractions 7-8) and HD (fractions 9-11) fractions were calculated by densitometry and were expressed as percentage of total signal assessed in the histogram. Patterns are representative of those obtained in three independent experiments. Data are the mean values ± S.D. of three different experiments. *, $p < 0.05$ *versus* control. $^{\#}$, $p < 0.05$ *versus* L-PDMP treated cells.

Effect of PDMP treatment on the association of c-Src and integrin receptor subunits with caveolin-1 in A2780/SAT-I cells

Caveolin-1, an integral membrane protein originally discovered as a main structural component of caveolae, has the potential to act as a molecular organizer for ganglioside-modulated signaling complexes. Several pieces of evidence indicate that caveolin-1 has the ability to interact with several proteins involved in signal transduction and to concentrate whole signaling modules in specialized plasma membrane areas, allowing their functional regulation. Moreover caveolin-1 has a phosphorylation site, tyrosine 14, which regulates Src activation. Caveolin-1 can also act as a membrane adapter, coupling the integrin receptor to cytosolic Src-family kinases. Results described in the previous section suggested that ganglioside depletion due to PDMP treatment, determines caveolin-1 dissociation from gangliosides/caveolin complexes and the subsequent association of caveolin-1 with integrin heterodimers. To confirm this hypothesis , we performed immunoprecipitation experiments using a polyclonal anti-caveolin-1 antibody. A2780/SAT-I cells, which have a high content of gangliosides and an elevated expression of caveolin-1, were pre-treated with 20 μM D- or L-PDMP for 48 hours before being collected and lysed in a solution containing Triton X-100. After a step of centrifugation to remove nuclei and cellular debris, we proceeded with the immunoprecipitation experiments as described in the Materials and Methods section.

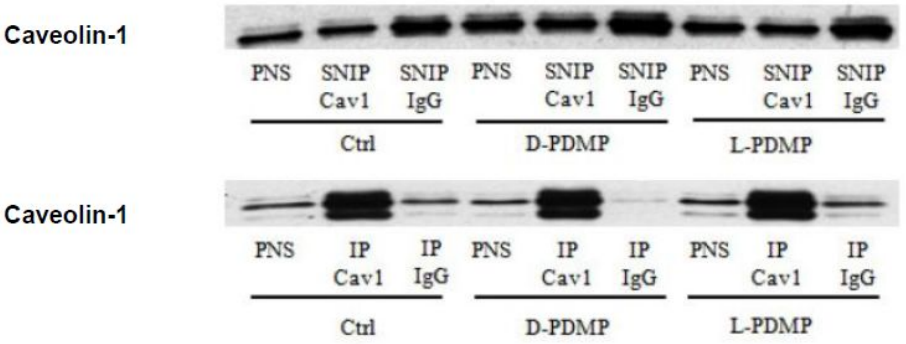
The presence of proteins such as caveolin-1, c-Src, integrin α5, and integrin β1 was detected by immunoblotting with specific antibodies.

As shown in figure 16 and 17, the caveolin-1 protein levels in the treated and untreated (control) cells were similar in the immunoprecipitate samples, indicating that the immunoprecipitation efficiency, which is about 15%, was the same for the three groups (Figure 16 and 17,A). The detection of c-Src, in the immunoprecipitate samples, revealed similar levels of the protein kinase after treatment with either D- or L-PDMP indicating that total levels of Src were not affected by the treatment (Figure 16,B). Integrin α5 levels in the immunoprecipitate samples, on the other hand, increase significantly after D-PDMP treatment, but not after the treatment with the inactive stereoisomer (Figure 16,C and 17,B). This confirms the previous hypothesis suggesting that D-PDMP treatment favors association of α5 with caveolin-1.

D-PDMP treatment also determines an increase of the percentage of integrin β1 in the immunoprecipitate. In particular, there is an increase of the mature form of integrin β1 which suggests that this form is the one that combines with caveolin-1 and takes part in the formation of the signaling complex. As shown in figure 16, the detection of β1 in the immunoprecipitate of the three groups revealed the presence of three bands, whereas only two bands are detected in the post nuclear surnatant. The lowest of the three bands represents a partially glycosylated precursor form of β1 that resides in the endoplasmic reticulum, while the middle one represents a further glycosylated form which is the functional receptor. Regarding the highest band there are two hypotheses, it could an aspecific band and thus not β1, or it might be a form of β1 which in the PNS is too low to be detected and that is concentrated upon caveolin-1 immunoprecipitation, which would mean that this form interacts preferentially with caveolin-1.

PDMP treatment did not affect the total levels of the protein analyzed, though D-PDMP treatment, but not L-PDMP treatment, determined an increase in the levels of the precursor form of integrin β1. This might be due to an aspecific effect of D-PDMP, which could inhibit sialyltransferases involved in the process of maturation of integrin β1.

A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SNIP IgG
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L-PDMP
Caveolin-1
PNS
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IP IgG
Ctrl
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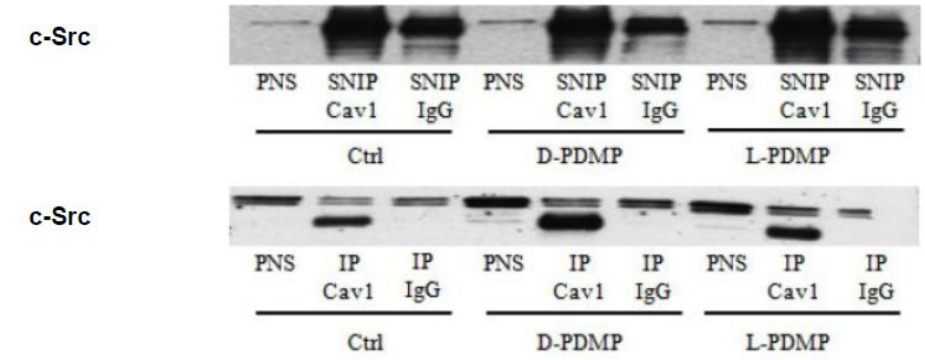
L-PDMP

B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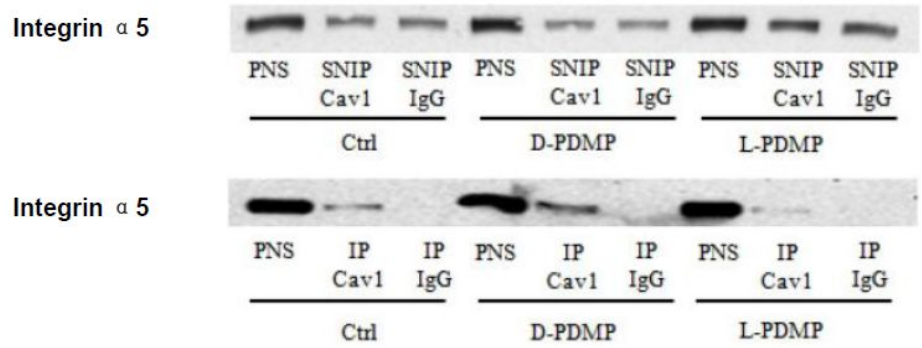
C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PNS
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IP IgG
Ctrl
D-PDMP
L-PDMP

D

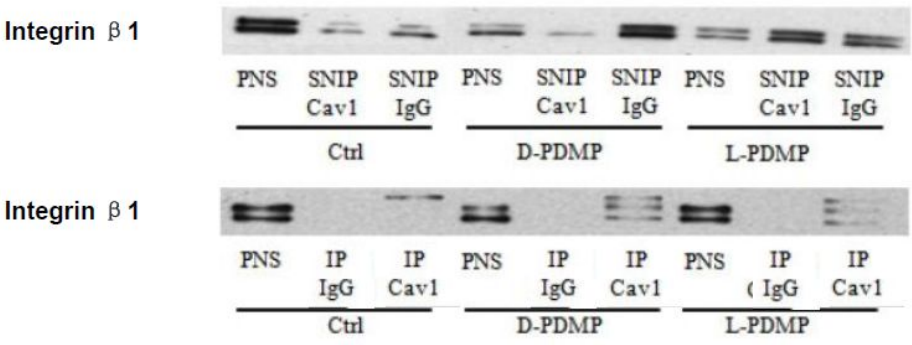
Integrin β 1
PNS
SNIP Cav1
SNIP IgG
Ctrl
D-PDMP
L-PDMP
Integrin β 1
PNS
IP IgG
IP Cav1
Ctrl
D-PDMP
L-PDMP

Figure 16. Effects of PDMP treatment on proteins associated with anti-caveolin-1 immunoprecipitates in SAT-I transfected cells. A2780/4T cells treated with D- or L-PDMP for 48 hours were collected and lysed in a solution containing Triton X-100. Immunoprecipitation experiments were performed on aliquots of the postnuclear surnatant (PNS) using a polyclonal anti-caveolin-1 antibody or normal rabbit IgG, as a negative control, as described in *Materials and methods*. Patterns are representative of those obtained in three independent experiments. ***Panel A***, for the detection of caveolin-1 we loaded 1/10 of total volume for Preclear samples, 1/580 of total volume for SNIP samples, and 1/35 of total volume for IP samples. ***Panel B***, for the detection of c-Src we loaded 1/10 of total volume for Preclear samples, 1/580 of total volume for SNIP samples, and 1/8 of total volume for IP samples. ***Panel C***, for the detection of integrin β1 we loaded 1/10 of total volume for Preclear samples, 1/580 of total volume for SNIP samples, and 1/5 of total volume for IP samples. ***Panel D***, for the detection of integrin α5 we loaded 1/10 of total volume for Preclear samples, 1/580 of total volume for SNIP samples, and 1/7 of total volume for IP samples.

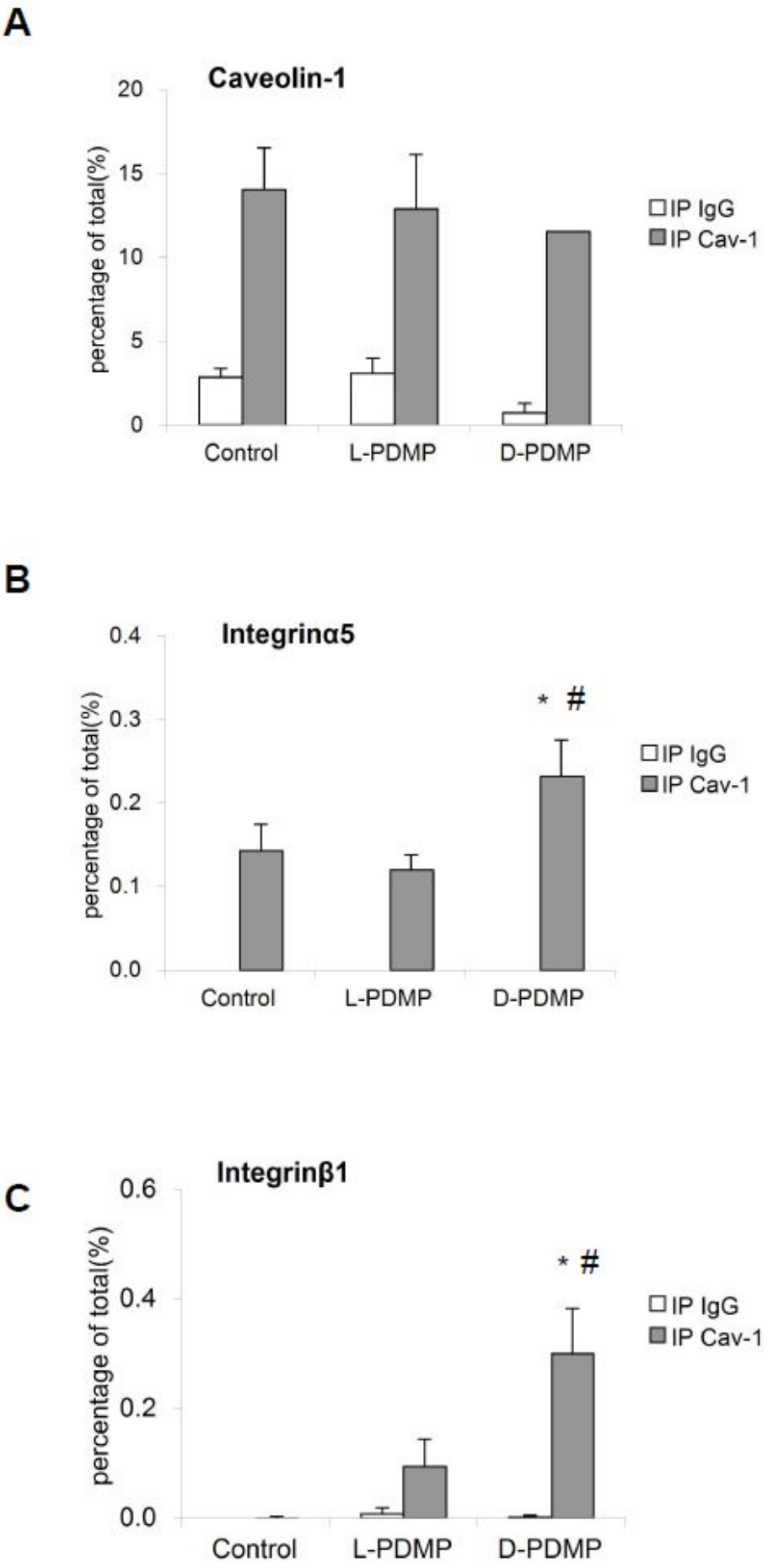
A
Caveolin-1
20
15
10
5
0
percentage of total(%)
IP IgG
IP Cav-1
Control
L-PDMP
D-PDMP
B
Integrinα5
0.4
0.3
0.2
0.1
0.0
percentage of total(%)
* #
IP IgG
IP Cav-1
Control
L-PDMP
D-PDMP
C
Integrinβ1
0.6
0.4
0.2
0.0
percentage of total(%)
* #
IP IgG
IP Cav-1
Control
L-PDMP
D-PDMP

Figure 17. Effects of PDMP treatment on proteins associated with anti-caveolin-1 immunoprecipitates in SAT-I transfected cells. The results reported in figure 16 were quantitatively expressed in bar graphs. The presence of caveolin-1, integrin α5 and integrin β1 in IP (anti-caveolin-1, grey; anti-IgG, white) samples was revealed by western blotting with specific antibodies. Data are expressed as percentage of total amount of each protein in 1.2 mg PNS used for immunoprecipitation and are the means ± S.D. of three different experiments. *, $p < 0,05$ *versus* control, untreated cells. $^{\#}$, $p < 0,05$ *versus* L-PDMP treated cells.

Effect of Transient silencing of caveolin-1 on *in vitro* cell adhesion in A2780/SAT-I cells

Since the levels of caveolin-1 expression were elevated in GM3 synthase overexpressed A2780 cells, if caveolin-1 is involved in ganglioside GM3 mediated cell adhesion, is worthy to be determined. Before that, if the transfection influences the cell proliferation levels need to be checked. The cells were previously silenced with the siRNA targeting to caveolin-1 gene, and then cell proliferation levels were measured by MTT reduction assay as described before. There was no difference between caveolin-1 silenced cells and scramble sequence transfected cells in the rate of cell growth (data not shown).

According to this result, *in vitro* adhesion of caveolin-1 silencing could be assessed in A2780/SAT-I cells. Caveolin-1 was silenced by siRNA transfection, and after 48 hours the adhesion to fibronectin was performed as described in *Materials and methods*. The *in vitro* adhesion of A2780/SAT-I transfected cells (Figure 18) was markedly higher in caveolin-1 silenced cells compared with scramble sequence transfected cells, suggesting a leading role of caveolin-1 in the regulation of the cell adhesion signal in this cell model.

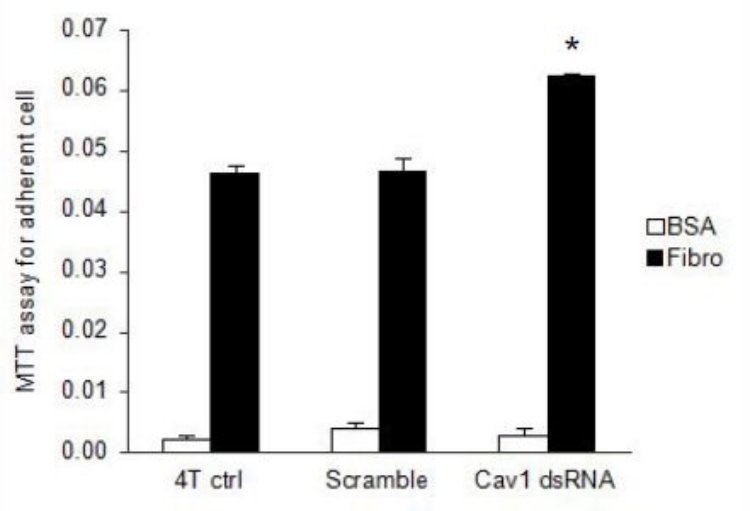

Figure 18. Effects of caveolin-1 silencing on *in vitro* cell adhesion in A2780/SAT-I cells. A2780/SAT-I-transfected A2780 cells have been treated with siRNA targeting to CAV1 gene to reduce the expression of caveolin-1. After 48 h treatment with scrambled siRNA or siRNA targeting to CAV1, cell adhesion assay was performed as described in the materials and methods section. Data are expressed in percentage respect to control cells treated with scrambled siRNA, and are the means ± S.D. of three independent experiments. *, $p < 0.001$ versus control, cells treated with scrambled siRNA.

Effects of exogenous administration of gangliosides on caveolin-1 expression and caveolin-1 phosphorylation level

Integrins connect the ECM to the actin cytoskeleton at special structures called focal adhesions (FA) through a protein complex that includes vinculin, paxillin, tallin and α-actinin (232). In addition, activation of integrins results in the recruitment of a number of signalling molecules to FA, including focal adhesion kinase (FAK). Caveolin-1 protein is also found at FA, where most of the phosphorylated caveolin-1 pool resides (104,233). Tyrosine phosphorylated Cav1 (pY14Cav1) appear to be essential for maintaining a highly ordered state in the membranes around these adhesion complexes, and this is likely to be due to the recruitment of membrane components that induce order, such as cholesterol (234). Besides its structural role, caveolin-1 also participates in active signalling at FA. In response to various stimuli, Src and other kinases phosphorylate caveolin-1 on Tyr 14, and this phosphorylation is crucial for a number of

functions attributed to caveolin-1. pY14Cav1 has also been reported to regulate Src activity by recruting C-terminal Src kinase (Csk) to FA (235,236). Csk inhibits Src activity by phosphorylating a conserved Tyr residue (243).

Since silencing of caveolin-1 by siRNA influenced the existence of pY14 Cav-1 on the cell plasma membrane as published before (5), implied that maybe not only caveolin-1 was involved in gangliosides mediated cell signals, but also the caveolin-1 phosphorylation plays a certain role in this regulation. The tyrosine phosphorylation site 14 (pY14) of caveolin-1 is considered as the active form of caveolin-1. Moreover, it is the major phosphorylation site of c-Src *in vitro*. Although its functional importance is still unclear, the following study is trying to reveal the role of pY14 Cav-1 under ganglisodies regulation. A2780 cells were exogenously administrated with gangliosides under experiment condition. The cells were lysed for detecting caveolin-1 and pY14 Cav-1 expression. As shown in Figure 15, only ganglioside GM3 treatment slightly increased caveolin-1 expression. Meanwhile, treatment of ganglioside GM3, GM2 and GM1, but not GD1a, significantly increased the phosphorylation levels of caveolin-1 normalized by β-tubulin. mRNA levels of caveolin-1 were also analyzed by semi-quantitative PCR and the results of mRNA levels showed the similar tendency as the protein levels (data not shown). This data recalled us to the effect of gangliosides treatment on the cell motility and adhesion in A2780 cells. It confirmed again that caveolin-1 participated in gangliosides modulating cell signaling, and it suggested that gangliosides mediated A2780 cell motility and adhesion through caveolin-1 phosphorylation.

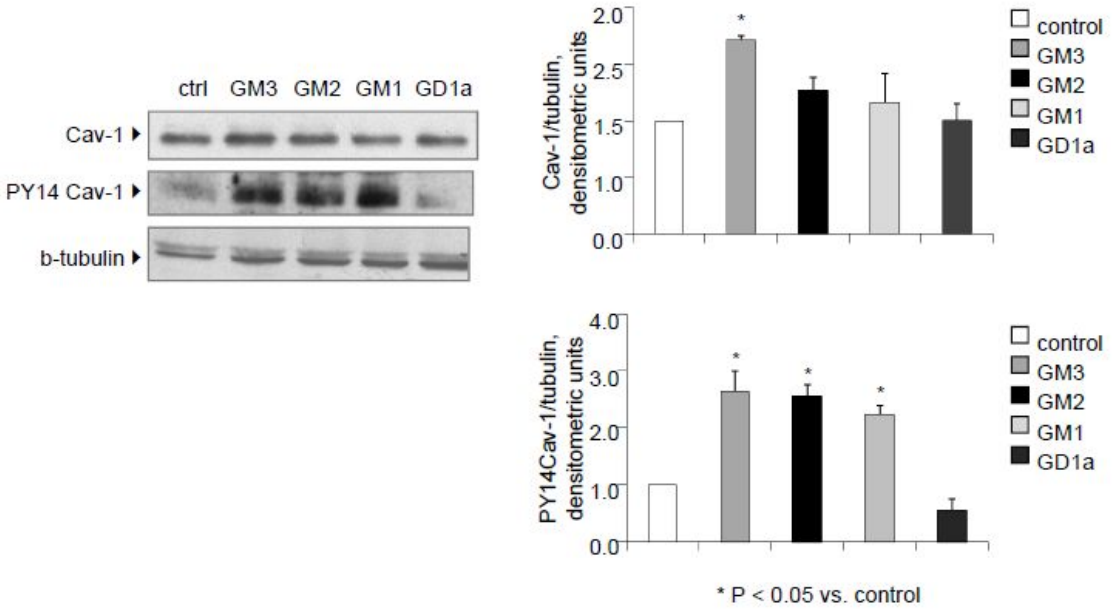

Figure 19. Effects of exogenous administration of gangliosides on caveolin-1 expression and caveolin-1 phosphorylation level. A2780 cell confluent monolayers were administrated with vehicle (control) or 50 μM of GM3, GM2, GM1, or GD1a for up to 48 h. After that, cells were analyzed by Western blotting detection using specific antibodies against caveolin-1 and Cav-1-pY14. β-tubulin was simultaneously detected as a loading control. Patterns are representative of those obtained in three independent experiments (left panel). The amounts of caveolin-1 and Cav-1-pY14 present in each sample were determined by densitometry, normalized respect to β-tubulin respectively, and expressed as a percentage of control (right panel). Data are the means ± S.D. of three different experiments, *, $p < 0.01$ versus controls, cells treated with vehicle only.

Effects of PDMP treatment on caveolin-1 expression and caveolin-1 phosphorylation level

In order to demonstrate that pY14 Cav-1 takes part in gangliosides regulation of A2780 cell motility and adhesion, A2780/SAT-I 4T cells were treated with L- or D-PDMP for 24 hours or 48 hours. After the treatment, cells were lysed and analyzed by Western blotting with specific antibodies against caveolin-1 and pY14 Cav-1. As shown in

Figure 20, both L- and D-PDMP treatment had no effect on caveolin-1 expression. However, D-PDMP, but not L-PDMP treatment was able to reduce caveolin-1 phosphorylation levels in A2780/SAT-I cells in a time dependent manner. Thus, in A2780 cell model, the phosphorylation level of caveolin-1 is related to gangliosides patterns, further indicating that gangliosides modulation of A2780 cells through caveolin-1 phosphorylation.

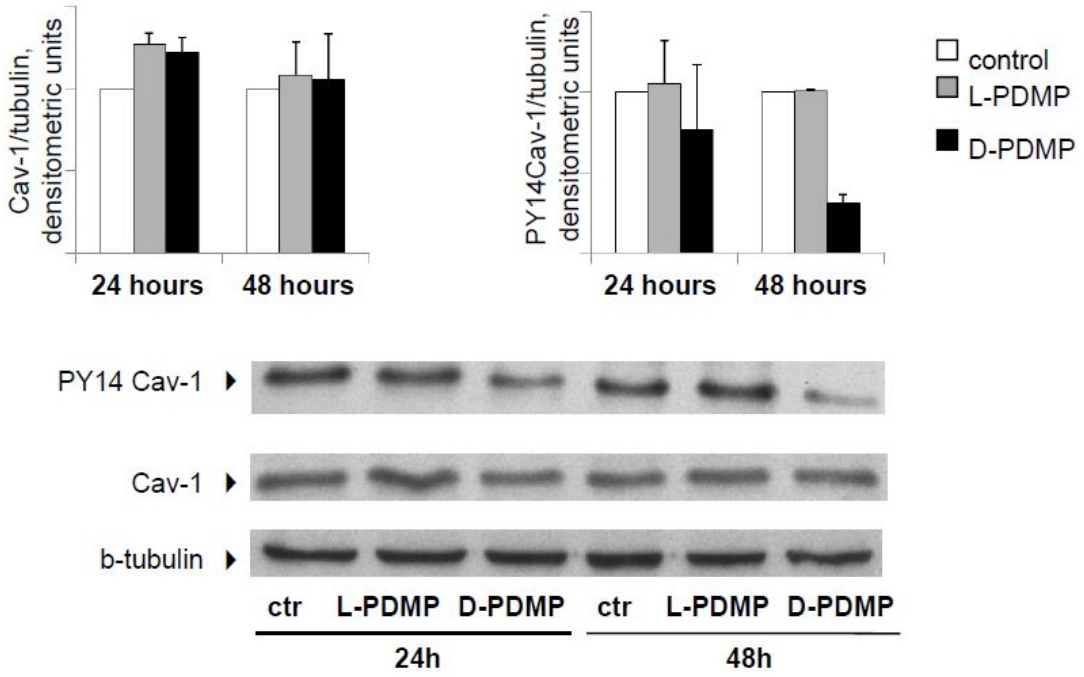

Figure 20. Effects of PDMP treatment on caveolin-1 expression and caveolin-1 phosphorylation level. A2780/SAT-I 4T cells were treated with 20 μM L- or D-PDMP for 24 and 48 hours. Total cell lysate from control or PDMP treatment cells were analyzed by Western blotting detection using specific antibodies against caveolin-1 and Cav-1-pY14. β-tubulin was simultaneously detected as a loading control. Patterns are representative of those obtained in three independent experiments (lower panel). The amount of caveolin-1 and Cav-1-pY14 present in each sample were determined by densitometry, normalized respect to β-tubulin respectively, and expressed as a percentage of time-matched controls (upper panel). Data are the means ± S.D. of three independent experiments. *, $p < 0.01$ *versus* controls, cells treated with vehicle only.

DISCUSSION

The data reported in this thesis and our previously published results (4,5), indicate that, in human ovarian cancer cells, an increase in cellular gangliosides content, caused by the overexpression of GM3 synthase, is paralleled by a marked up-regulation of the membrane protein adaptor caveolin-1. The result of these concomitantly high levels of gangliosides and caveolin-1 is a marked reduction of *in vitro* cell motility and an increase of adhesion.

Genetic manipulation of GM3 synthase levels by its stable overexpression in the case of SAT-I transfected cells, or the selective pressure in the presence of a drug (N-(4-hydroxyl)retinamide) in the case of A2780/HPR cells, allowed to obtain two cellular models in which higher GM3 synthase activity, compared with wild type cells, determined an up-regulation of caveolin-1, a reduced cell motility, and an increased cell adhesion. The existence of a causal connection between high levels of gangliosides and the observed reduction of cell motility/increased adhesive ability is supported by experiments in which exogenous gangliosides were administered to wild type cells, and also by depletion experiments, in which the ganglioside content was markedly reduced upon usage of the glucosylceramide synthase inhibitor, D-PDMP. The pharmacological inhibition of glucosylceramide synthase led to a strong enhancement of cell motility and a reduction of the cell adhesion. High ganglioside levels, therefore, are necessary, but not sufficient, to up-regulate cell adhesion and, in turn, down-regulate cell motility.

Moreover it has been shown that cells with a higher activity of GM3 synthase, also have a higher expression of caveolin-1, and the silencing of this protein led to an altered cell adhesion. Thus, the regulation of cell adhesion requires a certain degree of cooperation between gangliosides and caveolin-1. Caveolin-1, a hydrophobic membrane protein, was originally described as the main structural component of *caveolae*, omega-shaped invaginations of the plasma membrane that form a subdomain of cholesterol- and sphingolipid-rich lipid rafts and that are morphologically distinct from the triskelion structure of clathrin-coated pits (95). Soon though this protein gained a role as a molecular organizer for multiprotein signaling complexes, due to its ability to interact with several proteins involved in signal transduction through its scaffolding domain, and to concentrate whole signaling modules in specialized plasma membrane areas, allowing their functional regulation. Caveolin-1 is insoluble in cold non-ionic detergents (237), and can be enriched in low density, Triton X-100-insoluble membrane fractions. These fractions, which are also highly enriched in cholesterol and sphingolipids, putatively correspond to lipid rafts. Thus, caveolin-1 at the plasma membrane is

concentrated in a lipid-rich membrane environment, and lipids affect several of the functionally relevant properties of caveolin-1. Caveolin-1 and sphingolipids not only co-localize in the same detergent-resistant membrane fraction, but several pieces of evidence indicate that they can be close enough in specialized membrane subdomains to allow a direct interaction between the transmembrane domain of caveolin-1 and the hydrophobic moiety of the lipids (227,238). In SAT-I transfected A2780 ovarian carcinoma cells, photoreactive GM3 is able to label caveolin-1 in a detergent resistant membrane preparation (4). In a few cases, it has been also reported that detergent-resistant association of caveolin-1 and sphingolipids is strong enough to allow co-immunoprecipitation. In CHO cells transiently transfected by GD3 synthase cDNA, caveolin-1 can be immunoprepitated by a monoclonal antibody to ganglioside GD3 (239).

It has been reported that caveolin-1 and gangliosides are enriched in a Triton X-100 insoluble fraction and that a significant portion of the sphingolipids associated with the DRM fraction can be recovered upon immunoprecipitation with anti-caveolin-1 antibody (5). It has therefore been hypothesized that the formation of a ganglioside/caveolin-1 complex, occurring in cells with concomitantly high levels of both components, might be involved in the negative regulation of ovarian carcinoma cell motility. Several pieces of evidence indicate that integrin receptor subunits and the non-receptor tyrosine kinase c-Src are enriched together with caveolin-1 and sphingolipids within detergent-resistant membrane fractions. Moreover, integrin receptor subunints and c-Src co-immunoprecipitate with caveolin-1 as shown in figure 16 and 17. Caveolin-1 (6,7) and c-Src (240,241) are typically associated with sphingolipid-enriched membrane domains. In addition, in has been suggested that caveolin-1 might act as a membrane adapter, coupling integrin receptor to Src kinases (191) and that caveolin-1 mediated inactivation of the integrin/Src/FAK pathway might be responsible for the inhibition of metastatic potential in melanoma (242). Previously published data from our group strongly support the hypothesis that the inactivation of c-Src by ganglioside/caveolin-1 complex might result in the downregulation of ovarian carcinoma cell motility. We have previously shown that a Src inhibitor was able to inhibit the motility of A2780 wild type cells, which express low GM3 synthase and low caveolin-1, and that c-Src was less active in SAT-I transfected cells, expressing high caveolin-1 levels (4).

Moreover, we noticed that reducing the ganglioside content by D-PDMP treatment did not influence the expression of caveolin-1, c-Src and integrin, but altered the distribution of these molecules. As shown in figure 15, Caveolin-1 moved from the DRM fraction to the intermediate fraction, whose composition and biological function are still unclear. c-Src showed a distribution pattern similar to caveolin-1. Integrin α5 and integrin β1, shifted from the high density fraction to both DRM and intermediate fractions. These results imply that the change in the ganglioside composition resulted in the shift of molecules related to ganglioside signaling. It has also been shown that D-PDMP treatment can also reduce caveolin-1 motility, thus we supposed that caveolin-1 may bind to other molecules in the case of D-PDMP treatment. Since caveolin-1 and both integrin α5 and integrin β1 tend to move to the intermediate fraction after ganglioside depletion, integrin α5 and integrin β1 are possible candidates to associate with caveolin-1, thus slowing down caveolin-1 motility after D-PDMP treatment. Immunoprecipitation experiments with a polyclonal anti-caveolin-1 antibody supported this hypothesis showing an increase of both integrin α5 and integrin β1 associated with the caveolin-1 immunoprecipitate. In the case of integrin β1, the increase is referred to its mature fully glycosylated form, suggesting that this form is the one that interacts with caveolin-1 and takes part in the formation of the signaling complex.

Investigating *in vitro* cell adhesion to different extracellular matrix proteins, allowed us to hypothesize the presence of other integrin subunits involved in the regulation of the adhesion of A2780 ovarian carcinoma cells. The adhesion of both A2780 wild type and SAT-I transfected cells, not only to fibronectin which bind to integrin α5β1, the major integrin type expressed in these cells, but also to laminin and vitronectin suggest that integrin subunints such as αVβ1 and αVβ5, which bind vitronectin, or α3β1 and α6β1, which bind laminin, may have a role in regulating adhesion and motility, maybe by participating to the organization of a signaling complex involving caveolin-1. Vitronectin though also competes with fibronectin for its binding to α5β1, and it has been shown that Src family tyrosine kinases are required for fibronectin receptor α5β1 and vitronectin receptor αVβ5-mediated cell adhesion (243). Taken together, all these data suggest a novel role of gangliosides in the regulation of cell motility and adhesion in human ovarian carcinoma cells, by affecting the organization of a signaling complex organized by caveolin-1 and integrin α5, responsible for c-Src inactivation.

Our results call the attention to other aspects that probably deserve future consideration. First, despite the much higher expression of caveolin-1, the morphological analysis

revealed the absence of *caveolae* both in A2780/HPR and A2780/SAT-I transfected cells, as in the wild type A2780 cells. This finding might seem in contrast with the usual association between caveolin-1 and *caveolae*, but it is indeed in agreement with the diverse and multiple *caveolae*-independent roles of caveolin-1 that have been described in the last few years (6,7,244). The second aspect is related to the observation that cellular ganglioside or GM3 synthase levels can regulate the expression of caveolin-1. It has been shown that GD1a ganglioside regulated caveolin-1 expression in FBJ mouse osteosarcoma cells (245). Also in that case, the concomitant increase in certain gangliosides and in caveolin-1 seems to be related to the possible role of caveolin-1 as a tumor suppressor. FBJ mouse osteosarcoma cells and A2780 human ovarian carcinoma cells exist in different phenotypic variants, characterized by strikingly different *in vitro* cell motility. For both cell types, the low motility variants are characterized by high ganglioside and low caveolin-1 expression, while the high motility variants contain low ganglioside and low caveolin-1 levels. Treatment of high motile FBJ-LL osteosarcoma cell line with exogenous GD1a upregulation of caveolin-1 expression with reduced metastatic potential and suppressed cell adhesion to vitronectin (246). Similarly, GM3 synthase-transfected A2780 ovarian carcinoma cells were characterized by an increased expression of caveolin-1 and reduced *in vitro* cell motility. These cells also show a markedly increased *in vitro* cell adhesion to fibronectin, whereas the increase in their adhesion to laminin and vitronectin is not so striking. However, the mechanism underlying the regulation of caveolin-1 expression by the cellular gangliosides levels is totally unknown. The relationship between ganglioside and other molecules related to this signaling pathway needs to be further investigated.

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