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Role of gangliosides in modulating the motility of human 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.

Alterations in the structures of carbohydrate epitopes associated with glycosphingolipids are a common feature of tumors and tumor cells ("aberrant glycosylation"). In particular, tumors are characterized by the peculiar ability to manipulate sialylation processes (1). This abnormal sialylation process generates peculiar antigenic determinants, which are normally absent in healthy cells, and affects cell homeostasis, altering the normal signaling pathways. Indeed, glycosphingolipids in tumor cells have been implicated in the regulation of cell adhesion, motility, recognition, survival and proliferation (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 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).

Administration of exogenous gangliosides was able to strongly reduce *in vitro* cell motility and to increase cell adhesive ability to fibronectin in wild type cells, which

are low GM3 synthase-expressing A2780 cells. Conversely, in high GM3 synthase-expressing clones, ganglioside depletion by treatment with the glucosylceramide synthase inhibitor D-PDMP was able to strongly increase cell motility and to reduce adhesion. In these cells, transient silencing of caveolin-1 by siRNA also led to increased motility. Thus, high levels of caveolin-1 and high levels of gangliosides are necessary, but not sufficient, if independent, to down-regulate tumor cell motility.

Treatment of A2780 cells with exogenous gangliosides only slightly increased the expression of caveolin-1; on the other hand 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 motility.

The non-receptor tyrosine kinase c-Src plays a crucial role in controlling the motility of these cells. In fact, 1) the motility of low GM3 synthase-expressing cells was reduced in the presence of a Src inhibitor; 2) c-Src was less active in high GM3 synthase-expressing clones; 3) D-PDMP treatment of high GM3 synthase-expressing cells led to c-Src activation, while gangliosides administration in wild type cells, low GM3 synthase-expressing A2780 cells reduced c-Src kinase activity.

In high 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. In the presence of D-PDMP treatment, the distribution of several lipids in sucrose gradient changed, followed by a shift of both caveolin-1 and c-Src from DRM fraction to intermediate fraction. However, integrins, which are receptors that mediate attachment between cells moved from high density fraction to DRM and intermediate fraction.

All of these data suggest a novel role of gangliosides in regulating tumor cell motility, by affecting the organization of a signaling complex organized by caveolin-1,

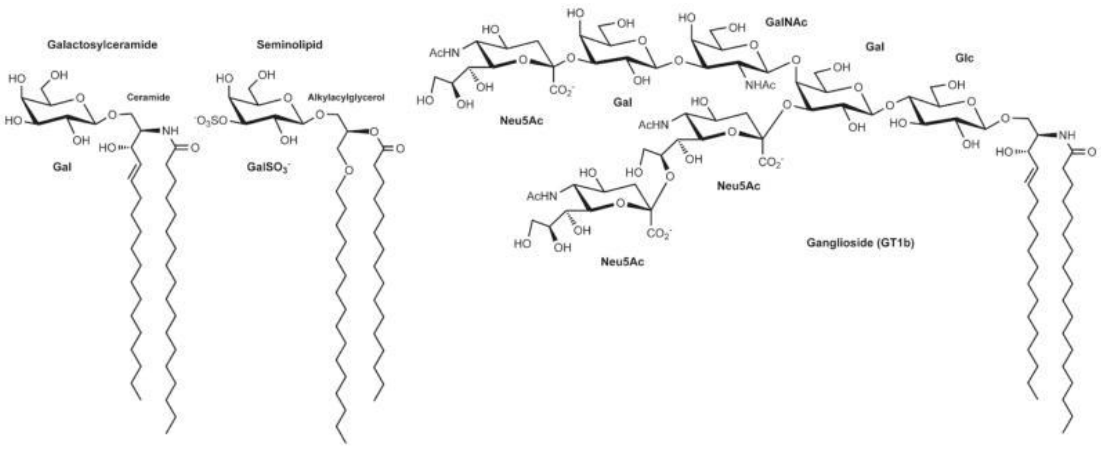
responsible for Src inactivation downstream to integrin receptors, and imply that GM3 synthase is a key target for the regulation of cell motility and adhesion in human ovarian carcinoma.

INTRODUCTION

Glycosphingolipids

Overview of glycosphingolipids

Glycerophospholipids, sphingolipids, and cholesterol are the lipid components of cell membranes. Among these, sphingolipids are minor components. They belong to the external layer of the membrane (8) with the hydrophilic headgroup protruding toward the extracellular environment. Glycosphingolipids are a subtype of glycolipids containing the amino alcohol sphingosine. Alternatively, they may be considered sphingolipids with a carbohydrate head group (9-11). Glycosphingolipids are ubiquitous components of mammal cell membranes, but are particularly abundant in the nervous system (12). Since sphingolipids are concentrated at the subcellular level in the plasma membrane, where they reside asymmetrically in the extracellular leaflet, they represent relatively abundant components in this district (13,14). Keeping in mind that sphingolipids are not homogeneously distributed throughout the membrane plane, but rather they are further concentrated in restricted membrane areas (15) due to their spontaneous segregation respect to glycerophospholipids, it can be predicted that their local concentration in specific “lipid membrane domains” would be very high (16). In these lipid domains, glycosphingolipids modulate the functional features of several membrane proteins through direct specific lipid-to-protein interactions or through the maintenance of a dynamic membrane organization. Thus, these complex membrane lipids participate in the modulation of processes, such as cell proliferation (17), survival (18), adhesion (19), and neuronal differentiation (20,21). 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) emerged as a class of lipid mediators capable to modulate cell proliferation, differentiation, motility or apoptotic cell death.

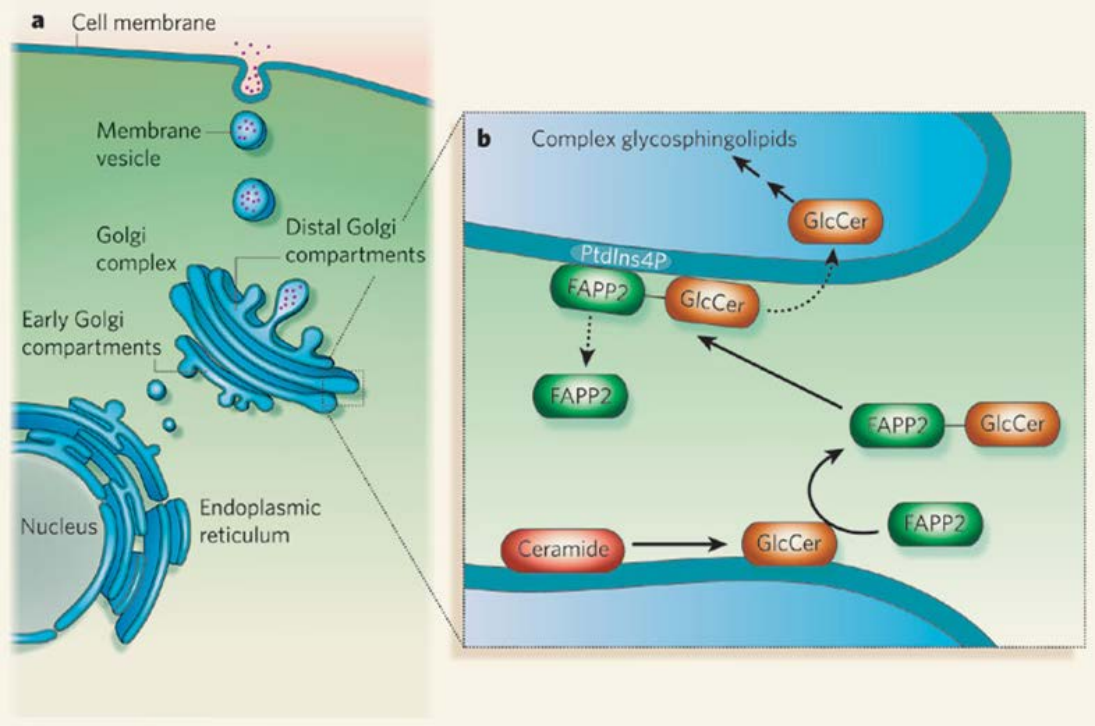


Structures of representative glycosphingolipids and glycoglycerolipids. Glycosphingolipids, such as GalCer, are built on a ceramide lipid moiety that consists of a long-chain amino alcohol (sphingosine) in amide linkage to a fatty acid. In comparison, glycoglycerolipids, such as seminolipid, are built on a diacyl or acylalkylglycerol lipid moiety. Most animal glycolipids are glycosphingolipids, which have a large and diverse family of glycans attached to ceramide. Shown is one example of a complex sialylated glycosphingolipid, GT1b ($IV^3NeuAcII^3[NeuAc]_2Gg_4Cer$).

Glycosphingolipids biosynthesis, trafficking and degradation

Glycosphingolipids biosynthesis occurs in a stepwise fashion, with an individual sugar added first to ceramide and then subsequent sugars transferred by glycosyltransferases from nucleotide sugar donors (22). Ceramide is synthesized on the cytoplasmic face of the endoplasmic reticulum (ER); it subsequently equilibrates to the lumenal face and trafficks to the Golgi compartment (23). GlcCer is synthesized on the cytoplasmic face of the ER and early Golgi apparatus; it then flips into the Golgi lumen, where it is typically elongated by a series of glycosyltransferases (24). In contrast, GalCer is synthesized on the lumenal face of the ER and then trafficks through the Golgi, where it may be sulfated to form sulfatide (25). In both cases, the final orientation of glycosphingolipids during biosynthesis is consistent with their

nearly exclusive appearance on the outer leaflet of the plasma membrane, facing the extracellular milieu. Although ceramide resides on intracellular organelles such as mitochondria, glycosphingolipids beyond GlcCer are not known to exist on membranes facing the cytoplasm (26,27).

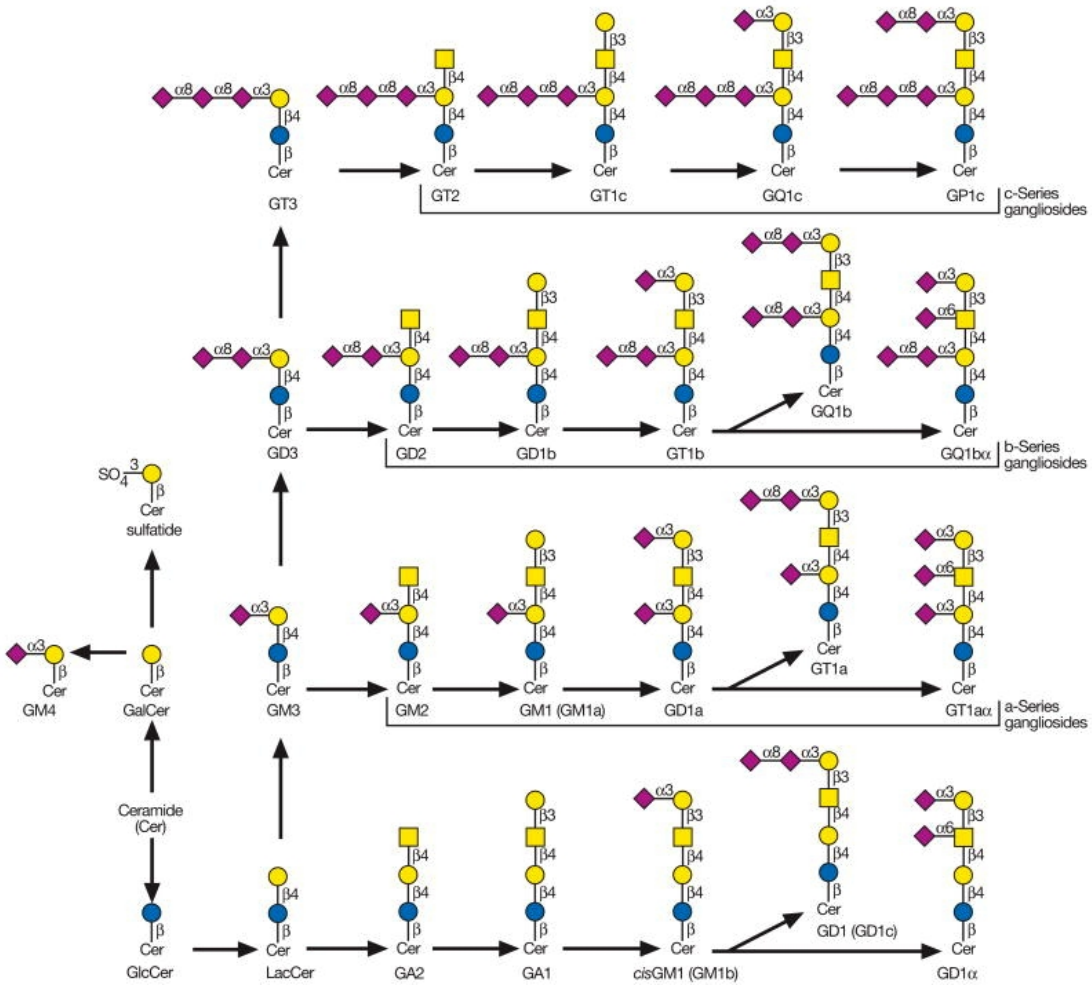


Glycosphingolipids biosynthesis. a, Most lipids are synthesized in the endoplasmic reticulum, and are then transferred to the Golgi complex. **b**, Glucosylceramide (GlcCer), which is synthesized from ceramide in the Golgi complex, has to be carried to more distal Golgi compartments for processing into complex glycosphingolipids. FAPP2 protein mediates transport of GlcCer between Golgi compartments. It picks up GlcCer from the membrane of one Golgi compartment and, on reaching its destination, binds to the membrane by interacting with phosphatidylinositol 4-phosphate (PtdIns4P). Consequently, GlcCer is released and translocated into the lumen of this compartment.

The biosynthesis of glycosphingolipids in the brain provides an example of how competing biosynthetic pathways can lead to glycan structural diversity (28). In the brain, stepwise biosynthesis of GalCer and sulfatide occurs in oligodendrocytes, the cells that elaborate myelin. Gangliosides, in contrast, are synthesized by all cells, with concentrations of the different forms varying according to cell type. Expression

patterns of glycosphingolipids are determined by the expression and intracellular distribution of the enzymes required for their biosynthesis. In some cases, multiple glycosyltransferases (29) compete for the same glycosphingolipid precursor. For example, the ganglioside GM3 may be acted on by *N*-acetylgalactosaminyltransferase (30), thereby forming GM2, the simplest of the “a-series” gangliosides, or by sialyltransferase (31), thereby forming GD3, the simplest of the “b-series” gangliosides. Each branch is a committed pathway, because sialyltransferases cannot directly convert a-series gangliosides (beyond GM3) to their corresponding b-series gangliosides. Due to this branch exclusivity, competition between two enzymes at a key branch point determines the relative expression levels of the final glycosphingolipid products. The transfer of *N*-acetylgalactosamine to a-, b-, and c-series gangliosides, transforming GM3 into GM2, GD3 into GD2, or GT3 into GT2, is catalyzed by the same *N*-acetylgalactosaminyltransferase. Likewise, the transfer of galactose to GM2 to form GM1, to GD2 to form GD1b, or to GT2 to form GT1c is accomplished by a single galactosyltransferase. An additional level of regulation may occur via stable association of different glycosphingolipid glycosyltransferases into functional “multiglycosyltransferase” complexes. The multiple enzymes are then thought to act concertedly on the growing glycosphingolipid without releasing intermediate structures, ensuring progression to the preferred end product.

The breakdown of glycosphingolipids occurs stepwise by the action of lysosomal hydrolases (32,33). Glycosphingolipids on the outer surface of the plasma membrane are internalized, along with other membrane components, in invaginated vesicles that then fuse with endosomes, resulting in the glycosphingolipid glycan facing the endosome lumen. Glycosphingolipid-enriched areas of the endosomal membrane may then invaginate once again to form multivesicular bodies within the endosome. When endosomes fuse with primary lysosomes, glycosphingolipids become exposed to lysosomal hydrolases. In vivo, glycosphingolipids are eventually broken down to their individual components, which are then available for reuse.



Biosynthetic pathway for brain glycosphingolipids. Glycosphingolipids are synthesized by the stepwise addition of sugars first to ceramide, then to the growing glycan. Shown as examples are brain glycosphingolipids. Ceramide (Cer) is the acceptor for UDP-Gal:ceramide β-galactosyltransferase or UDP-Glc:ceramide β-glucosyltransferase in the major pathways to glycosphingolipid biosynthesis in oligodendrocytes and nerve cells, respectively. GalCer is the acceptor for GalCer sulfotransferase, which adds a sulfate group to the C-3 of galactose to form sulfatide. Extension of GlcCer to the major brain gangliosides occurs by the action of UDP-Gal:GlcCer β1-4 galactosyltransferase to make lactosylceramide (LacCer), then CMP-NeuAc:lactosylceramide α2-3 sialyltransferase to make the simple ganglioside GM3. GM3 is a branch point and acts as the acceptor for UDP-GalNAc:GM3/GD3 β1-4 *N*-acetylgalactosaminyltransferase to generate a-series gangliosides and for CMP-NeuAc:GM3 α2-8 sialyltransferase to generate GD3 and the b-series gangliosides. Similarly, the action of an α2-8 sialyltransferase on GD3 gives rise to GT3 and the c-series gangliosides. Enzymes for subsequent elongation are common to the a-, b-, and c-series gangliosides.

Glycosphingolipids functions

Glycosphingolipids are essential for the survival, proliferation, and differentiation of eukaryotic cells within complex multicellular systems. Glycolipid-deficient cells, such as the GM-95 mutant melanoma cell line, lacking ceramide glucosyltranferase activity (34), and embryonic stem cells from ceramide glucosyltranferase knockout mice (35) are able to survive, grow, and undergo *in vitro* differentiation. However, ceramide glucosyltranferase knockout mice are embryonic lethal, and showed no cellular differentiation beyond the primitive germ layers (36). These observations indicate the vital importance of glycosphingolipids in the life of cells that are dealing with a multifaced extracellular reality.

As mentioned above, glycosphingolipids are not randomly distributed along the membrane surface, but they are rather highly segregated together with cholesterol in lipid domains with specialized signaling functions (15). A high local concentration of glycosphingolipids in the plasma membrane has important implications with regard to their ability to engage both *trans* and *cis* functional interactions with other cellular components. In the case of *trans* interactions, it has been shown that recognition of lipid-bound oligosaccharides by soluble ligands (such as 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) (37). On the other hand, *cis* interactions, i.e., direct lateral 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 (38).

Glycosphingolipid patterns undergo deep qualitative and quantitative modifications during the development of the nervous system, and along differentiation in cultured neurons (39-46). A widely used experimental model for the study of glycosphingolipid biological functions in intact cells or in membrane preparations rely on the administration of exogenous gangliosides dissolved in the culture medium. The binding, uptake and metabolic fate of exogenous gangliosides under different

experimental conditions have been well characterized (47,48). 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 (42,49-51).

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 (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, D-PDMP, and analogues) (52) or by inhibitors of sphinganine *N*-acyltransferase (the enzyme that catalyses the synthesis of dihydroceramide, the biosynthetic precursor of ceramide and of all complex sphingolipids) (53), reduced axonal elongation and branching in cultured hippocampal and neocortical neurons (54-56), and NGF-induced neurite outgrowth in human neuroblastoma and PC12 cells (57,58). Conversely, up-regulation of glycosphingolipid biosynthesis by L-PDMP stimulated neurite outgrowth in cultured cortical neurons (56,59). In the same cellular model, D- and L-PDMP exerted as well opposite effects on the formation of functional synapses and on synaptic activity (59).

As mentioned above, the role of glycosphingolipids 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 (60-62). These interactions are usually highly specific. In the case of receptor-associated tyrosine kinases, well-studied examples are represented by epidermal growth factor receptor (EGFR), whose tyrosine phosphorylation and dimerization are inhibited by GM3, but uninfluenced by GM1 (63), and by insulin receptor, inhibited by GM3 but not by GD1a (64). On the other hand, many papers indicated that the colocalization of glycosphingolipids and signaling proteins within sphingolipid- and

cholesterol-enriched membrane domains might imply a functional link even in the absence of direct strong and specific glycosphingolipid-protein interactions, suggesting that overall lipid raft dynamics, as determined by their peculiar lipid composition, might be rather responsible for the functional modulation of raft-associated signaling proteins (15,65-69).

Glycosphingolipids and tumor

Glycosphingolipids play important roles in modulating several properties of tumor cells. Indeed, a correlation between the expressions of some carbohydrate structures associated with glycolipids and tumor patient survival rates has been observed, and elevated serum ganglioside levels have been reported in patients (2,70). On the other hand, in tumor cell lines, the tumorigenic potential correlates with the cellular levels of gangliosides (71-73), and the ability to form experimental tumors can be affected by the artificial manipulation of cellular ganglioside levels (74). 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 (75-78), and cross-talk of EGFR with integrin receptors (79) and PKCα (80), 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 (81) and Met (82,83) 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 (84,85), and the overexpression of GM3 synthase reduced cell proliferation, motility, and invasion in mouse bladder carcinoma cells (74). In colorectal (86) and bladder (85) 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 (81). On the other hand, tetraspanin CD82 is essential for the ganglioside-mediated cross-talk of EGFR with other signaling pathways (80). 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.

Caveolin-1

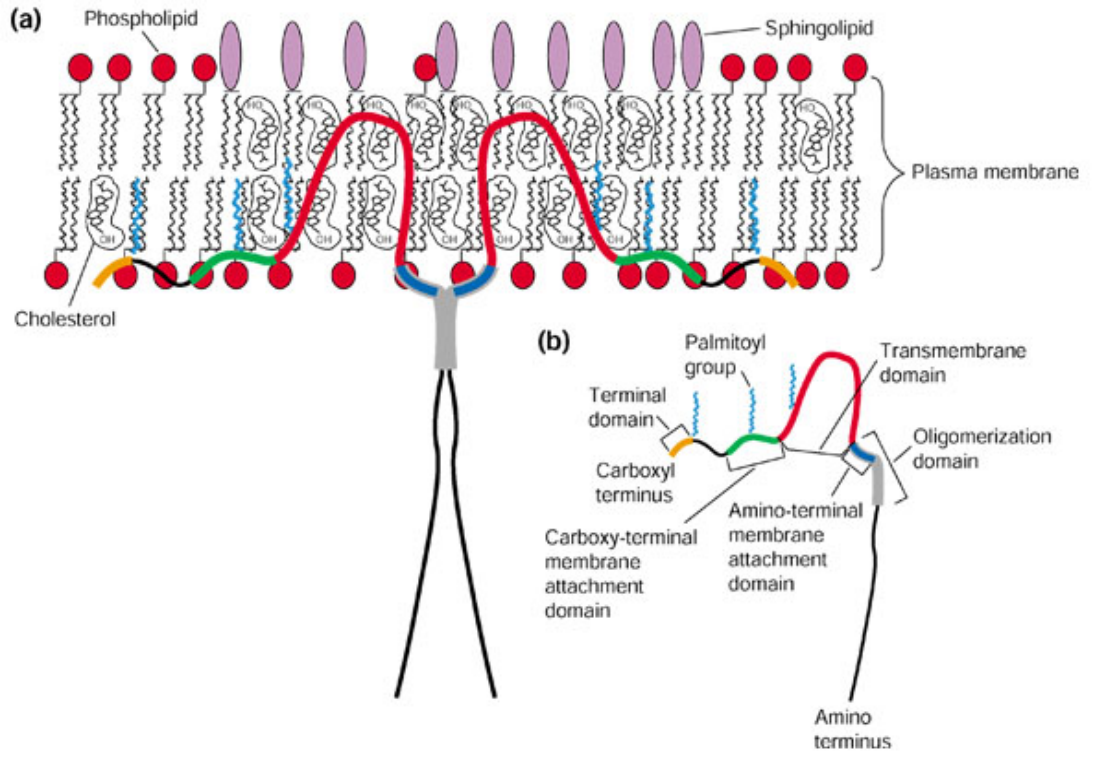
Caveolin-1 structure

Caveolins (87,88) are a family of 21- to 24-kDa integral membrane proteins, that have been originally described as the main structural protein components of plasma membrane specializations known as *caveolae* (89,90). Three distinct caveolin genes, which encoding caveolin proteins individually: caveolin-1, caveolin-2 and caveolin-3, have been identified (91). While caveolin-3 is found mainly in skeletal muscle fibers and cardiac miocytes (92), caveolin-1 and caveolin-2 are co-expressed in most cells (they are highly expressed in adipocytes, endothelial cells and fibroblasts) and share many physical properties, although the latter seems to lack full functional capacity to form *caveolae*. Caveolin-1 has a hydrophobic putative membrane-spanning sequence and it is palmitoylated at the C-terminal domain. Caveolins form high mass oligomeric complexes, providing a scaffold for caveolin interacting proteins (including H-Ras (93), c-Src, heterotrimeric G proteins (93,94) and growth factor receptors (95,96)), that can thus be concentrated within caveolin-rich membrane areas (97,98).

Caveolin-1 as a negative regulator of 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. In certain cell types, antisense inhibition of caveolin-1 expression is sufficient to induce the oncogenic transformation. Targeted downregulation of caveolin-1 in NIH-3T3 cells activates MAPK and stimulates anchorage-independent growth (99). Loss of caveolin-1 is required to accelerate tumorigenesis and metastasis: PyMT/Cav-1 (-/-) mice showed accelerated onset of mammary tumors and lung

metastasis (100). Caveolin-1 gene (CAV-1) is markedly down regulated in human tumors derived from the ovary, breast and colon, but it is up regulated in tumor samples from the kidney, prostate and stomach.

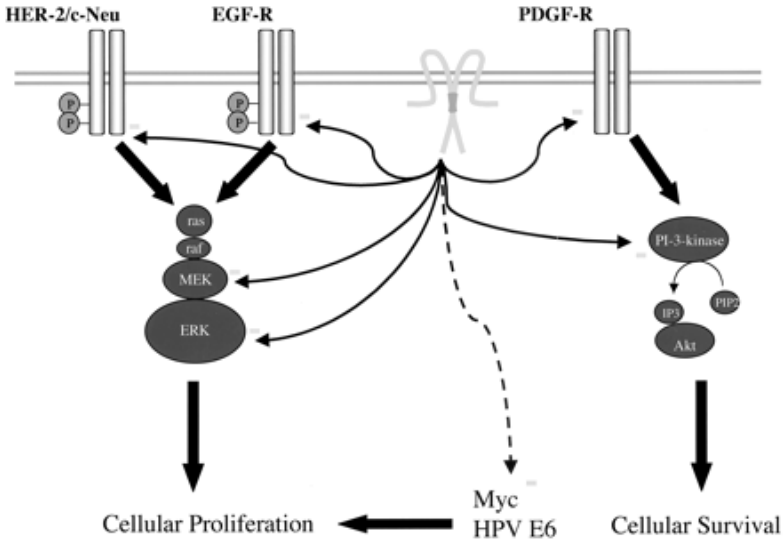


Primary structure and topology of caveolin-1. (a) The predicted membrane topology of caveolin-1. Two caveolin-1 monomers are shown forming a dimer for simplicity, but about 14-16 monomers normally self-associate to form a single caveolin homo-oligomer (the caveolar assembly unit, akin to the clathrin triskelion). Note that both the amino- and carboxy-terminal domains are oriented towards the cytosolic face of the plasma membrane, with a hairpin loop structure inserted within the membrane bilayer. **(b)** The domains present in caveolin-1. Note that the amino-terminal membrane-attachment domain is also called the caveolin scaffolding domain (CSD).

Expression of caveolin-1 in a highly metastatic carcinoma-derived cell line suppressed lung metastasis *in vivo* and reduced Matrigel invasion *in vitro*. Decreased invasion in caveolin-1-expressing cells was accompanied by reduction in MMP9 and MMP2 secretion and gelatinolitic activity, and reduced ERK1/2 signaling in response to growth factors (100). Caveolin-1 potentially restrains tumor cell growth and

metastatic potential: caveolin-1 re-expression in human breast cancer and in colon carcinoma cell lines inhibited tumor cell growth (101), reduced tumorigenicity (102), 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 kinase (103).

On the other hand, there is an 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 (104-106). 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 the commitment to apoptosis induced by several cellular stressors (107). Interestingly, sphingomyelin, the precursor to ceramide generation, is one of the most abundant lipids in *caveolae*, and sphingomyelinase, the ceramide-generating enzyme, has been localized to *caveolae* microdomains (108). Furthermore, overexpression of caveolin-1 sensitizes cells to ceramide-induced cell death via a phosphatidylinositol 3-kinase-dependent mechanism (106). 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 (109).



Caveolin-1 negatively regulates signaling along several pro-proliferative and anti-apoptotic pathways. Consistent with a tumor-suppressor role, caveolin-1 can potently inhibit signaling originating from certain receptor tyrosine kinases (HER-2/c-Neu and EGFR) and some of their downstream components (including the Ras-p42/44 MAP kinase cascade). In addition, caveolin-1 has been shown to facilitate apoptotic signaling by shutting down certain members of the pro-survival phosphatidylinositol 3-kinase (PI-3-kinase)/Akt pathway. The other inhibitory functions of caveolin-1 (e.g., abrogation of Myc- or HPV E6-mediated transformation) are less well understood.

Caveolin-dependent signaling and glycosphingolipids

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: (1) 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 ganglioside (probably in noncaveolar membrane regions) allows the interaction of EGFR with activated PKC-α, ultimately leading to the inhibition of EGFR signaling (80,110-113). However, this signaling complex does not

seem to require a direct caveolin-GM3 interaction (80). (2) In other cases, caveolin-1 and sphingolipids can compete for a common interactor. This is exemplified by insulin receptor (IR), that can form a complex with caveolin-1 (probably in *caveolae*) required for insulin signaling leading to the translocation of GLUT4 at the surface of normal adipocytes (114). Accumulation of GM3 upon acquisition of insulin resistance leads to the displacement of IR from the caveolin-1 complex and its sequestration as a complex with GM3 (115). 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, which is from *caveolae* (116), 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 direct bind sphingolipids, including GM1, in this case it cannot be excluded that 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*.

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 (117). 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 steps 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 (118). Lipid raft-dependent (cholesterol- and sphingolipid-sensitive) but caveolin-1-independent internalization pathways have also been described. The picture is still fragmentary, but it becomes apparent that *caveolae*- and lipid raft-dependent endocytosis are similar but distinct processes, that are interdependent and reciprocally regulated (119,120). The situation is made even more complex by two relevant observations: (1) association of a molecule with lipid rafts/*caveolae* does not necessary implies 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 rafts (121-123); (2) usually the internalization of lipid raft components via the clathrin-mediated mechanism requires that they move outside *caveolae*/lipid raft 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 (124,125).

Under basal conditions, *caveolae* are relatively immobile structures with a low turnover at the plasma membrane levels (126), 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 phosphatase (127) and of major

histocompatibility complex class I (128), cell membrane attachment of SV40 virus (129), disengagement of integrin receptors upon cell detachment (130)). Based on the observations that the loss of *caveolae* does not impair endocytosis of some lipid raft markers (131,132) 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 (133), 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 (119,120). Triggering of *caveolae*/raft-mediated internalization would thus require additional factors allowing overcoming to 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 (134), and glycosphingolipids stimulate caveolar endocytosis (135,136). Glycosphingolipids could directly affect the membrane environment of caveolin-1, or could regulate tyrosine phosphorylation of caveolin-1, that is an essential requirement for caveolar/raft endocytosis (130). Indeed, using fluorescent sphingolipid analogues it has been proven that sphingolipid segregation in endocytic vesicles is essential for caveolar endocytosis. On the other hand, glycosphingolipid-stimulated caveolar endocytosis required Src activity, and addition of exogenous sphingolipids or cholesterol has been shown to stimulate Src activity (137). Thus, likely multiple mechanisms regulated by sphingolipids are potentially responsible for triggering caveolar endocytosis.

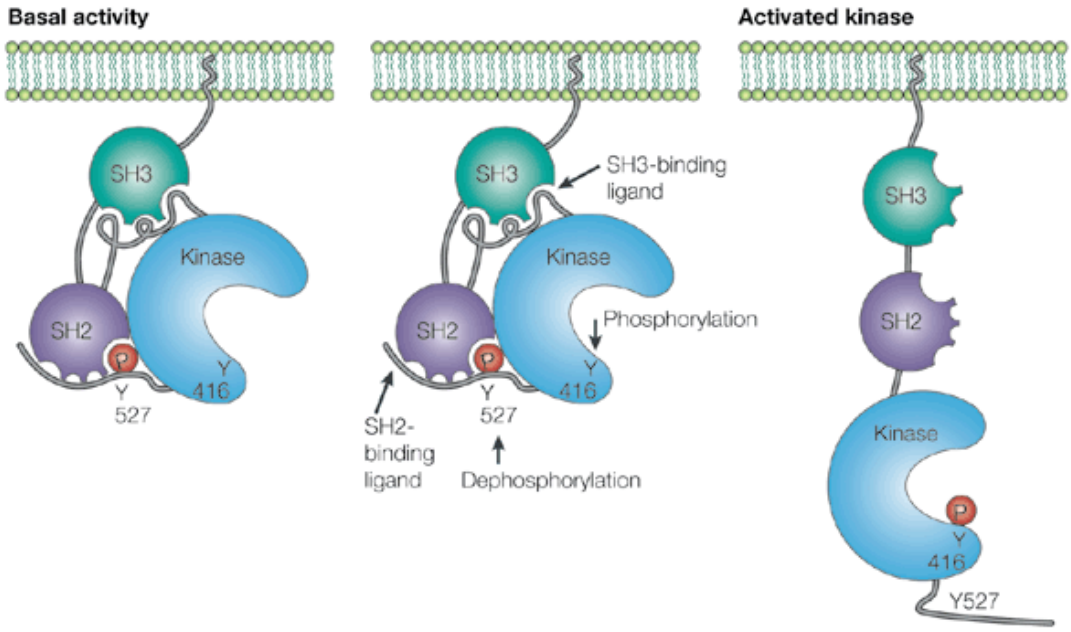
Along this line, another mechanism that could be involved in glycosphingolipid-regulated and caveolin-mediated clearance of plasma membrane receptors is suggested by the observation that many RTKs are at least in part localized in lipid rafts, have a caveolin binding motive and form complexes with caveolin-1, as discussed above. In all these cases, the elevation of cellular ganglioside levels has as a consequence the shift of the receptor outside of *caveolae*. In the case of IR and PDGFR, this resulted in the uncoupling of the receptor from the downstream

signaling cascade. However, as mentioned above, movement of RTK outside caveolar membrane domains potentially target these receptors to clathrin-dependent internalization pathways, thus contributing in the negative regulation of cell surface concentration of the receptor.

Src kinases

Src tyrosine kinases

The c-Src gene, discovered in the 1970s, is a “proto-oncogene” in normal mammalian cells. The protein product of c-Src gene (SRC) belongs to the Src family tyrosine kinases (SFKs), a group of non-receptor tyrosine kinases, which are involved in many of the signaling mechanisms associated with G-protein-coupled receptors, integrins, receptor tyrosine kinases, T-cell receptors, and others (138). Of the eight family members, c-Src, Yes, and Fyn are expressed ubiquitously, with the other members being expressed primarily in lymphocytes (139). 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 catalytic domain contains an autophosphorylated tyrosine (416 in c-Src), which is phosphorylated when the enzyme is active. SH2 domains bind phosphotyrosine motifs, and SH3 domains bind polyproline motifs. In the inhibited state, the SH2 domain of c-Src is involved in an intramolecular interaction with a C-terminal regulatory domain phosphotyrosine, tyrosine 527 (140), locking the enzyme in an inactive or closed state. Oncogenic activation in the case of v-Src results from the loss of this C-terminal regulatory domain (141). Phosphorylation of the C-terminal regulatory tyrosine on SFKs is catalyzed by C-terminal Src kinase (Csk) (142). Csk is required for normal development, because Csk knockout mice die at embryonic day 9 or 10 (143). The architecture of Csk is similar to SFKs with one SH2 domain, one SH3 domain and a kinase domain (144). It is known that Csk lacks a regulatory C-terminal tyrosine, N-terminal myristoylation, and membrane association domain (145). Although SFKs are membrane-associated and regulated by phosphorylation, Csk is intrinsically cytoplasmic (146) and requires membrane adaptors to inhibit membrane-associated SFKs.

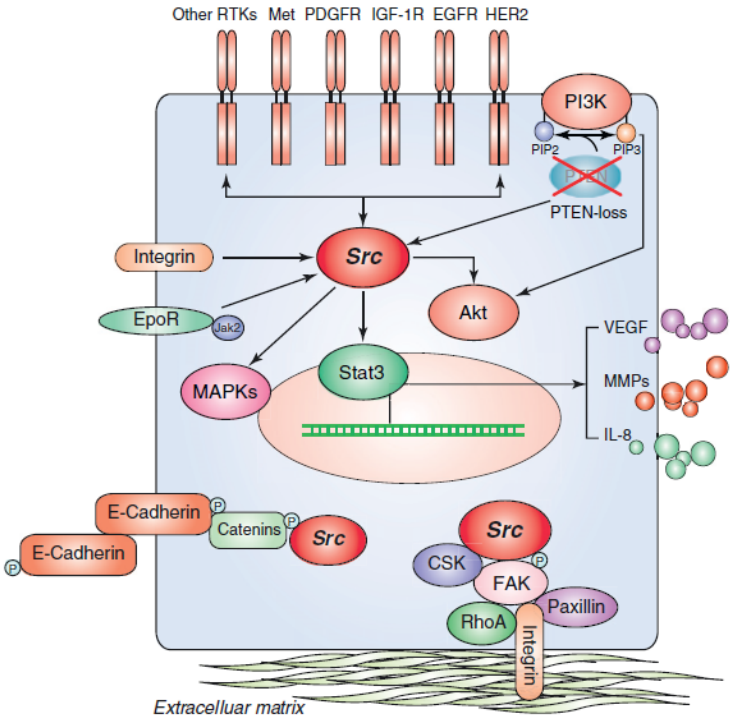


Proposed model for Src activation. The left panel represents the inactive conformation of Src, in which Tyr527 interacts with the SH2 domain, positioning the SH3 domain to interact with the linker between the SH2 and catalytic domains. The middle panel illustrates different mechanisms involved in the activation of Src, and the right panel represents the open or active conformation.

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 (147). 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 (148). Src has long been known to interact with epidermal growth factor receptor (EGFR) (149), human epidermal growth factor receptor 2 (HER2 or ErbB2) (150), platelet-derived growth factor receptor (PDGFR) (151), insulin-like growth factor-1 receptor (IGF-1R) (152) and c-Met/hepatocyte growth factor receptor (HGFR) (153). Through these interactions, Src integrates and

regulates RTK signaling and directly transduces survival signals to downstream effectors such as phosphoinositide 3-kinases (PI3Ks), Akt and signal transducer and activator of transcription 3 (STAT3) (154). Src can also be activated by other membrane receptors including integrins and erythropoietin receptor (EpoR) (147,155). 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 (155). Through interaction with p120 catenin, Src activation promotes dissociation of cell-cell adherens junctions and facilitates cell mobility (156). 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 (155). Additionally, Src also plays a part in regulating the tumor microenvironment. Under hypoxic conditions, Src activation promotes angiogenesis through stimulation of vascular endothelial growth factor (VEGF) (157), matrix metallopeptidases (MMPs) (158) and interleukin-8 (IL-8) expression (159). Src-mediated VEGF secretion elicits angiogenic signaling in endothelial cells and Src activation in osteoclasts facilitates osteolytic bone metastasis (147,155,160).



Src involved cell signaling pathways. The major downstream signaling upon Src activation include: (i) activation of Akt and

enhancement of cell proliferation; (ii) STAT3 activation and transcriptional upregulation of secretary factors involved in metastasis and angiogenesis, (e.g. matrix metallopeptidases (MMPs), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8); (iii) disruption of cell-cell adherens junctions through phosphorylation of p120-catenin; (iv) stabilization of focal adhesion complex through phosphorylation of FAK. epidermal growth factor receptor (EGFR); erythropoietin receptor (EpoR); focal adhesion kinase (FAK); human epidermal growth factor receptor 2 (HER2); insulin-like growth factor-1 receptor (IGF-1R); interleukin-8 (IL-8); Janus kinase 2 (Jak2); mitogen-activated protein kinase (MAPK); phosphatase and tensin homolog (PTEN); phospho-inositide 3-kinase (PI3K); platelet-derived growth factor receptor (PDGFR); receptor tyrosine kinases (RTKs); signal transducer and activator of transcription 3 (STAT3).

Caveolin-1 and c-Src

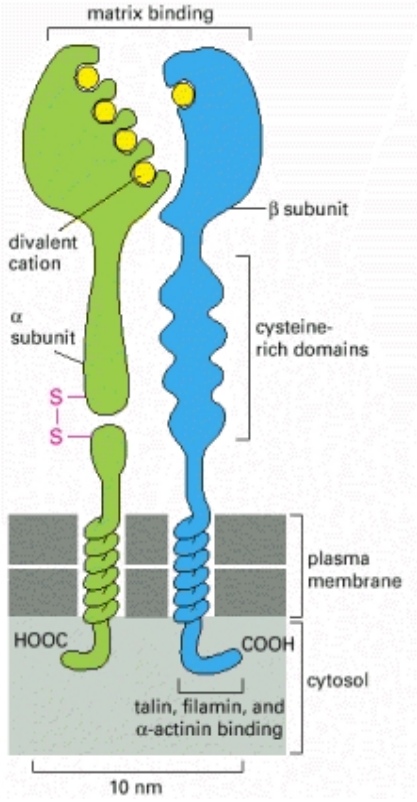
It has been recently shown that caveolin-1 promotes cell-cell adhesion in ovarian carcinoma cells by a mechanism involving inhibition of Src kinases (161). Non-receptor tyrosine kinases of the Src family are involved in several cell functions such as mitogenic response of growth factors (162-169), fibroblasts cell migration and epithelia cell scattering (166,170,171) and in cancers (172,173). 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). Src kinases are activated and involved in cancer progression and metastasis of most human carcinoma. Published 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 ganglioside 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 (173). Why Csk in colon cancer cells does not regulate efficiently Src kinases? 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. CBP/PAG phosphorylated on tyrosine 317 by activated Src kinases binds and activates Csk that in turn downregulates Src kinases. 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 receptors to Src kinases (174). Src induces phosphorylation of caveolin-1 at Tyr14, that is responsible for the rearrangement of caveolin-1 within the cell (175-177). 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 (178). 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 (179-181).

Integrins

Integrin structures

The linkage of the extracellular matrix to the cell requires transmembrane cell adhesion proteins that act as matrix receptors and tie the matrix to the cell cytoskeleton. Although we have seen that some transmembrane proteoglycans function as co-receptors for matrix components, the principal receptors on animal cells for binding most extracellular matrix proteins, including collagens, fibronectin, and laminins are the integrins. These constitute a large family of homologous transmembrane, cell-matrix adhesion receptors. An integrin molecule is composed of two noncovalently associated transmembrane glycoprotein subunits called α and β (182). 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 (183).



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 140,000-dalton 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.

Many matrix proteins in vertebrates are recognized by multiple integrins. At least eight types of integrins bind fibronectin, for example, and at least 5 types bind laminin. A variety of human integrin heterodimers are formed from nine types of β subunits and twenty-four types of α subunits (184). This diversity is further increased by alternative splicing of some integrin RNAs. Some of the best-studied integrins and their ligands are listed below:

Integrin	Ligands*	Distribution
$\alpha_5\beta_1$	fibronectin	ubiquitous
$\alpha_6\beta_1$	laminin	ubiquitous
$\alpha_7\beta_1$	laminin	muscle
$\alpha_L\beta_2$	Ig superfamily counterreceptors	white blood cells
$\alpha_2\beta_3$	fibrinogen	platelets
$\alpha_6\beta_4$	laminin	epithelial hemidesmosomes

*Not all ligands are listed.

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 (reviewed in (185)). A factor common to all these signaling events is that 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 the extracellular matrix (ECM). Activation of Rac 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 signalling (186,187). Thus, when β_1- and probably other fibronectin-binding integrins are uncoupled from downstream signaling by detaching cells from the ECM, PAK is not activated by Rac, even though Rac-GTP (activated by growth factor receptors) is present in these detached cells (187). These data suggest 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 (CEMMs): (1) association of Rac with CEMMs within the plasma membrane has been reported in several studies (130,188-191), including an unbiased proteomic approach (191); and (2) the loss of integrin signalling promoted by cell detachment induces a rapid internalization of CEMMs, and this prevents the targeting of Rac to the plasma membrane and its coupling to PAK. Replatement of cells on fibronectin or anti-β_1-integrin antibody reversed these effects (130). 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 (130).

Localization to CEMMs has also been described for the other two members of the Rho family of GTPases, Rho and Cdc42 (188,192-194). Rho and its effector mDia regulate microtubule stabilization (194), and this process occurs only at the leading edges of migrating cells, which are enriched in Rho and CEMMs (195). The local coupling between Rho and its effector is regulated by integrin-mediated cellular adhesion to the ECM, and appears to require active signalling by focal adhesion kinase (193). Cdc42 targeting to the plasma membrane is also integrin dependent (186). Therefore, evidence for association with CEMMs in an integrin-dependent manner has been described for all three major members of Rho family GTPases.

Glycosphingolipids and integrins

One mechanism by which glycosphingolipids could affect cell adhesion and migration is via their interaction with integrins. Integrins are a family of heterodimeric, integral membrane proteins at the plasma membrane, which bind to extracellular matrix (ECM) proteins and cell surface ligands, and are responsible for many types of cell adhesion events (196,197). Glycosphingolipids have been shown to directly modulate integrin-based cell attachment. For example, gangliosides (sialic acid-terminated glycosphingolipids) extracted from neuroblastoma cells or atherosclerotic plaques enhance platelet adhesion via integrin binding to collagen (198-200). Gangliosides also enhance binding of integrins to the ECM in mouse mammary carcinoma, melanoma, and neuroblastoma cells (201-203). Several models have been proposed for the mechanisms by which glycosphingolipids or glycosphingolipid-enriched microdomains may regulate integrin function (200,204,205). 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 the clustering of integrins in glycosphingolipid-enriched microdomains, thus increasing their avidity for ligand. The cross-linking of integrins with certain integrin antibodies is an established method for integrin activation (206,207). Similarly, integrin function can be modulated by antibody cross-linking of cholera toxin B subunit bound to GM1 ganglioside or glycophosphatidylinositol-linked proteins (204,205). 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* (207,208), a subset of glycosphingolipid enriched microdomains defined as invaginations at the plasma membrane enriched in caveolin-1 (209,210). *Caveolae* are sites for clathrin-independent endocytosis of glycosphingolipids as well as some viruses and bacteria toxins (135,137,211-214). It was reported recently that the addition of glycosphingolipids or cholesterol to the plasma membrane of cells stimulates caveolar endocytosis via activation of Src kinase (135). Treatment with exogenous sphingolipids the cells began to reorganize their actin cytoskeleton and retract, suggesting a link between plasma membrane glycosphingolipid and cholesterol composition and cell adhesion via integrins.

AIMS 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 regulate cell proliferation, mainly by affecting the tyrosine kinase activity associated with growth factor receptors (e.g. EGFR) and receptor compartmentalization, thus regulating the responsiveness of these receptors to their ligands or their cross-talk with other signaling modules. On the other hand, gangliosides 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. Ganglioside 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.

On the basis of these considerations, aims of the present study are 1) 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; 2) to explore the possible candidate molecules that are involved in gangliosides mediated signal transduction.

The objectives of this project will contribute significantly to the greater 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.

Lipids and radioactive lipids

GM1, GM2 and GD1a were prepared from the bovine brain ganglioside mixture and purified by partitioning (215). GM3 was prepared from GM1 using the GM1-lactone hydrolysis procedure (216). [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 (217).

Cell lines and culture

Human ovarian carcinoma cells A2780 were kindly provided by Dr. F. Formelli (Department of Experimental Oncology, National Cancer Institute, Milan, Italy) and were obtained by Dr. R.F. Ozols (National Cancer Institute, Bethesda, USA). The cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) that was purchased from GIBCO, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

The cell line A2780/HPR, resistant to the drug HPR (*N*-(4-hydroxyphenyl) retinamide), was obtained from the parental cells A2780 through *in vitro* administration with increasing sub-lethal concentrations of HPR (Sigma) (218). The HPR resistance in this cell is reversible. For this reason, the drug HPR was dissolved in dimethylsulfoxide (DMSO) to the storage concentration of 10 mM. A2780/HPR cells are constantly cultured in presence of 5 μM HPR.

The cell line A2780/SAT-I was also obtained from the parental cells A2780 through transfection with the cDNA of sialyltransferase-I (SAT-I), so-called GM3 synthase. The GM3 synthase expression vector (pSAT1-Rc/CMV) (a generous gift of Dr. I. Colombo, University of Milan, Milan, Italy) was constructed by subcloning SAT-I cDNA into an expression vector pRc/CMV (219). A2780 cells were transfected by cationic polymers (Open Biosystems, Huntsville, AL) with the pSAT1-Rc/CMV or with the empty vector, following the manufacturer's protocol. Stable transfectants were isolated after selection with 750 μg/ml of geneticin (G418, Sigma). In order to keep culturing the cells with GM3 synthase cDNA, A2780/SAT-I cells are constantly cultured in the presence of 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-Strand 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 genes GAPDH and ACTB were 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'
CAV1	5' –GAGCTGAGCGAGAAGCAAGT-3'	5'- TCCCTTCTGGTTCTGCAATC-3'
CAV2	5' –ACGACTCCTACAGCCACCAC-3'	5'-CGTCCTACGCTCGTACACAA-3'
ACTB	5'-CGACAGGATGCAGAAGGAG-3'	5'-ACATCTGCTGGAAGGTGGA-3'
GAPDH	5'-CGAGATCCCTCCAAAATCAA-3'	5'-GGTGCTAAGCAGTTGGTGGT-3'

GM3 synthase activity assay

Cells cultured in 100-mm dishes as described previously were harvested using a plastic scraper and washed two times with phosphate-buffered saline (PBS). Cells were resuspended in 150 mM sodium cacodylate-HCl buffer, pH 6.6 (20 mg of cell protein/ml) with protease inhibitors (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.0016 mM aprotinin, 0.044 mM leupeptin, 0.08 mM bestatin, 0.03 mM pepstatin A, 0.028 mM E-64) (Sigma) and homogenized with a Dounce homogenizer (10 strokes, tight). In each reaction tube, 10 µl of Triton CF-54 1.5% (v/v) in chloroform/methanol (2:1) was mixed with 0.5-50 nmol of [3-^{3}H(sphingosine)]LacCer (corresponding to 45 nCi) from a stock solution in chloroform/methanol (2:1) and dried under N_2. To this

mixture, 8 µl of 750 mM sodium cacodylate-HCl buffer, pH 6.6, 4 µl of 125 mM $MgCl_2$, 4 µl of 125 mM 2-mercaptoethanol, 10 µl of 5 mM CMP-NeuAc, and 10 µl of cell homogenate (containing 200 µg of protein) was added in a total reaction volume of 50 µl. Negative controls were performed using heat-inactivated cell homogenates (100 °C for 3 min). The incubation was performed at 37 °C for 3 h with continuous shaking. The reaction was stopped by adding 1.5 ml of chloroform/methanol (2:1). The reaction mixture was analyzed by HPTLC using the solvent system chloroform/methanol/water (55:20:3 by volume). Radioactive lipids were detected and quantified by radioactivity imaging as described below.

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. 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.

Cell adhesion assay

Cell adhesion to defined matrix components was accomplished as previously described (220). 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. 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, 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. Optical density (absorbance at 570 nm minus that at 650 nm) was measured to evaluate cells attached to the substrate.

Protein quantification with DC assay

The protein quantification was performed through Bio-rad™ DC assay. This DC assay is a colorimetric assay for protein concentration following detergent solubilization and is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent.

The protein standard used in this assay should be dissolved in the same solvent as the samples. Add 5 μl of each concentration of the standard, which is bovine serum albumin (BSA), to a 96 well in triple. Then 25 μl of reagent A and 200 μl reagent B, which were both supplied in Bio-rad™ DC assay kit, were added respectively in each well. Shake the plate with a rotator for 15 min and then the absorbance at 750 nm was measured with the spectrophotometer. The sample readings were compared with the ones of the standard. The assay is linear between 1.5 and 7.5 μg of protein amount.

Western blotting

Cell homogenates, gradient fractions and immunoprecipitation samples were analyzed by SDS-PAGE and immunoblotting using mouse monoclonal anti-Cav-1-pY14 (BD Transduction Laboratories), mouse polyclonal anti-integrin α5 (BD Transduction Laboratories), rabbit monoclonal anti-c-Src (Cell Signaling), rabbit polyclonal

anti-caveolin-1 (BD Transduction Laboratories), anti-Src-pY416 (Cell Signaling), anti-Src-pY527 (Cell Signaling) followed by reaction with secondary horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence detection (Pierce). β-actin or β-tubulin was used as loading control (anti-β-actin rabbit polyclonal antibody is from Santa Cruz, anti-β-tubulin mouse monoclonal antibody is from 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.

c-Src inhibition

A2780 cells grown in 100-mm culture dishes as confluent monolayers were pretreated for 6 h with 3 μM SU6656 Src inhibitor (Sigma) or with the equal volume of dimethyl sulfoxide (DMSO) (control), then confluent cell monolayers were wounded and cell motility was assessed by wound healing assay as described above. Cells were maintained in the presence of DMSO or 3 μM SU6656 for the duration of the migration assay.

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 [205]. 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.

Lipid extraction and determination

At the end of the treatment periods, cells adherent to the dishes were harvested in ice-cold water (2 ml) by scraping with a rubber policeman. In the case of gradient fraction, water was omitted. Cells floating in the culture medium were collected by centrifugation. Both adherent and floating cells were analyzed to determine the content of radioactivity associated with lipids. The samples were lyophilized, and lipids were extracted twice with chloroform/methanol 2:1 by volume (first extraction, 1.5 ml; second extraction, 0.25 ml). The total lipid extracts were subjected to a two-phase partitioning as previously described (222), resulting in the separation of an aqueous phase containing gangliosides and an organic phase containing all the other lipids.

Aliquots of total lipid extracts, aqueous and organic phases were analyzed by HPTLC. The phospholipid content was determined in the organic phase as phosphate after perchloric acid digestion by the method of Bartlett (223). The ganglioside content was determined as lipid-bound sialyc acid by the resorcinol method (224). Cholesterol was

quantified by visualization with 15% concentrated sulfuric acid in 1-butanol (217). The quantity of cholesterol was determined by densitometry and comparison with 0.1-2 μg of standard compounds.

The radioactivity associated with cells, lipids, lipid extracts, and aqueous or organic phases was determined by liquid scintillation counting. Radioactive lipids were detected and quantified by radioactivity imaging performed with a Beta-Imager 2000 instrument (Biospace, Paris, France) using an acquisition time of about 48 h. The radioactivity associated with individual lipids was determined with the specific β-Vision software provided by Biospace.

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

Phagokinetic assays with gold colloid-coated plates were performed as described (225). Briefly, 24-mm coverslips were coated with 1% bovine serum albumin (BSA) (Sigma-Aldrich) and then immersed in the colloidal gold solution (226,227). 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.

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 motility analysis by wound healing assay, cells were pre-treated with siRNA for 48 h before the assay, and siRNA administration was repeated after 48 h.

Immunofluorescence analysis of caveolin-1

A certain number of A2780 and A2780/HPR cells were plated on 24-mm round sterilized coverslips. A2780/HPR cells were transfected with scrambled siRNA or siRNA targeting CAV1 mRNA as described above. After 72 h, cells were first washed with PBS, then fixed with 4% paraformaldehyde in PBS and incubated for 10 minutes at room temperature. The coverslips were washed once with PBS and then the cells were permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Three washes with PBS were performed and then the aspecific binding sites were blocked through incubation with blocking buffer (10% horse serum, 1% BSA in PBS) for 1 hour at room temperature. After that, the coverslips were washed with PBS and incubated with the primary antibodies diluted in 1% BSA in PBS (dilution of 1:250 for the caveolin-1 antibody) overnight at 4° C. The following day the coverslips were washed three times with PBS and then incubated with FITC-conjugated secondary antibodies (anti-rabbit for caveolin-1) diluted 1:25 in 1% BSA in PBS for 45 minutes at room temperature avoid light. Three washes with PBS and one with water were performed and then the coverslips were dried very well. The coverslips were fixed on microscope slides with a drop of 90% glycerol and observed under an Olympus BX50 fluorescence microscope. The acquisition time of the fluorescence was the same for every sample. The slides were observed with a magnification of 100 × under the oil-immersion lens.

Src immunocomplex kinase assay

Cells were lysed in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 2 mM NaF,

1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/ml aprotinin). Protein concentration was adjusted to 1 µg/µl with RIPA buffer. 500 µg of proteins from each sample were used for immunoprecipitation. Samples were pre-cleared for non specific binding by adding 10 µl G protein-coupled magnetic beads (Dynabeads, Invitrogen). Precleared samples were incubated with 4 µg anti-Src antibody (clone GD11, Millipore, Temecula, CA) or normal rabbit IgG, previously adsorbed on 10 µl G protein-coupled beads following manufacturer's instruction for 1 h at 4 °C. The anti-Src immunoprecipitated samples, conjugated to the magnetic beads were recovered and incubated with 10 µCi [γ-^{32}P]ATP (Perkin Elmer) and 10 µM ATP in Kinase buffer (30 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM Na_3VO_4, 2 mM NaF, 1 mM DTT, 1 mM PMSF and 75 mU/ml aprotinin) at 37 °C for 1 h. The reaction was stopped by adding equal volume of ice-cold 2 × RIPA buffer, beads were washed with ice-cold RIPA buffer and boiled at 100 °C for 5 min with 2 × Laemmli buffer. The samples were separated by SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were analyzed by autoradiography by exposing to Kodak BioMax MR Film (Sigma-Aldrich) at -80 °C for certain time, or were acquired with a Beta-Imager 2000 instrument (Biospace, Paris, France) using the acquisition time of about 48 h. c-Src protein level was detected by western blotting as described above.

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

24 h after seeding, cells were incubated in the presence of 3 × 10^{-8} M [1-^{3}H]sphingosine (5 ml/dish) in culture medium for 2 h (pulse). After the pulse, the medium was replaced with fresh medium without radioactive sphingosine, and cells were further incubated for up to 4 days (chase). Under these conditions, all sphingolipids (including ceramide, SM, neutral glycolipids, and gangliosides) and phospholipids (obtained by recycling of radioactive ethanolamine formed in the

catabolism of [1-^{3}H]sphingosine) were metabolically radiolabeled (45,217,228).

Preparation of DRM fractions by sucrose gradient centrifugation

Cells were subjected to homogenization and to ultracentrifugation on discontinuous sucrose gradient, as previously described (217). 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. The radioactive lipids were analyzed as described above.

RESULTS

GM3 synthase overexpression in A2780 cells

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

As shown in Figure 1, the SAT-I mRNA levels (Panel A) and GM3 synthase activity measured *in vitro* on the natural substrate LacCer (Panel B) 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. The mRNA level of GM3 synthase in A2780/HPR cells was also detected. Both of the mRNA level of SAT-I and GM3 synthase activity were much higher compared to A2780 wild type cell, consistent with the previous result that the content of GM3 is higher in A2780/HPR cells than A2780 cells.

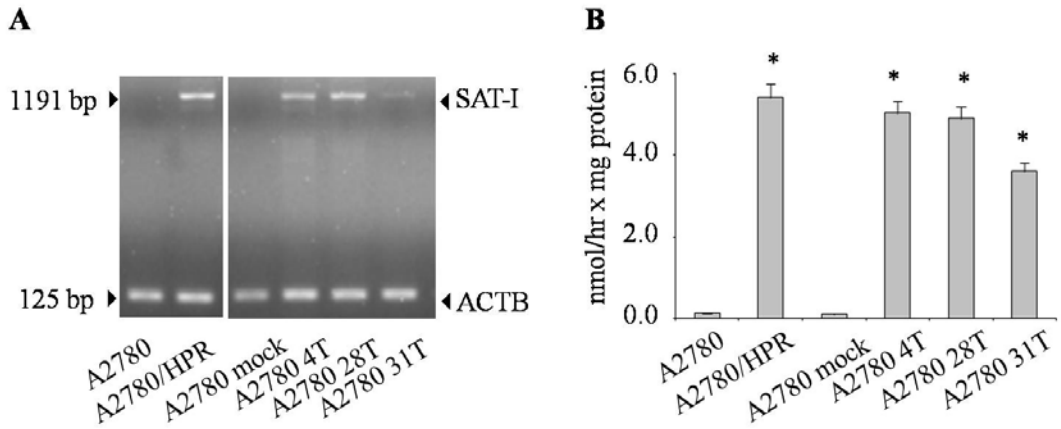


Figure 1. SAT-I expression in A2780/SAT-I transfected clones. SAT-I cDNA was cloned into a plasmid expression vector under the control of CMV promoter. A2780 cells were transfected with the empty expression vector (A2780 mock) or the vector containing the SAT-I cDNA; selection was performed in the presence of geneticin and, after 30 days, some colonies were subcloned and expanded.

(Panel **A**) SAT-I mRNA levels were assessed in A2780 and A2780 transfectant (one mock and 3 SAT-I-transfected clones, 4T, 28T, and 31T) cells by RT-PCR. ACTB mRNA expression was measured as an internal control. Patterns are representative of those obtained in three different experiments. (Panel **B**) *In vitro* GM3 synthase activities were measured on cell lysates using 100 μM radioactive LacCer as a substrate. Data are expressed as nmoles LacCer converted into GM3/h × mg cell protein, and are the means ± S.D. of three different experiments. *, $p < 0.01$ *versus* A2780 cells.

The lipids composition of transfectants were also evaluated (data not shown), indicating that phospholipids and cholesterol levels were not changed in cells with high GM3 synthase activity, while the ganglioside content was 2.2- and 2.0-fold higher in 4T and 28T SAT-I transfectants with respect to control cells, respectively. This increase in the ganglioside content in SAT-I transfectants was due to higher levels of GM3, GM2 and GD1a (229).

Effect of overexpression of SAT-I on the *in vitro* motility of A2780 cells

We investigated the effect of increased GM3 synthase expression levels on A2780 *in vitro* motility. 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 trasfectants (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.

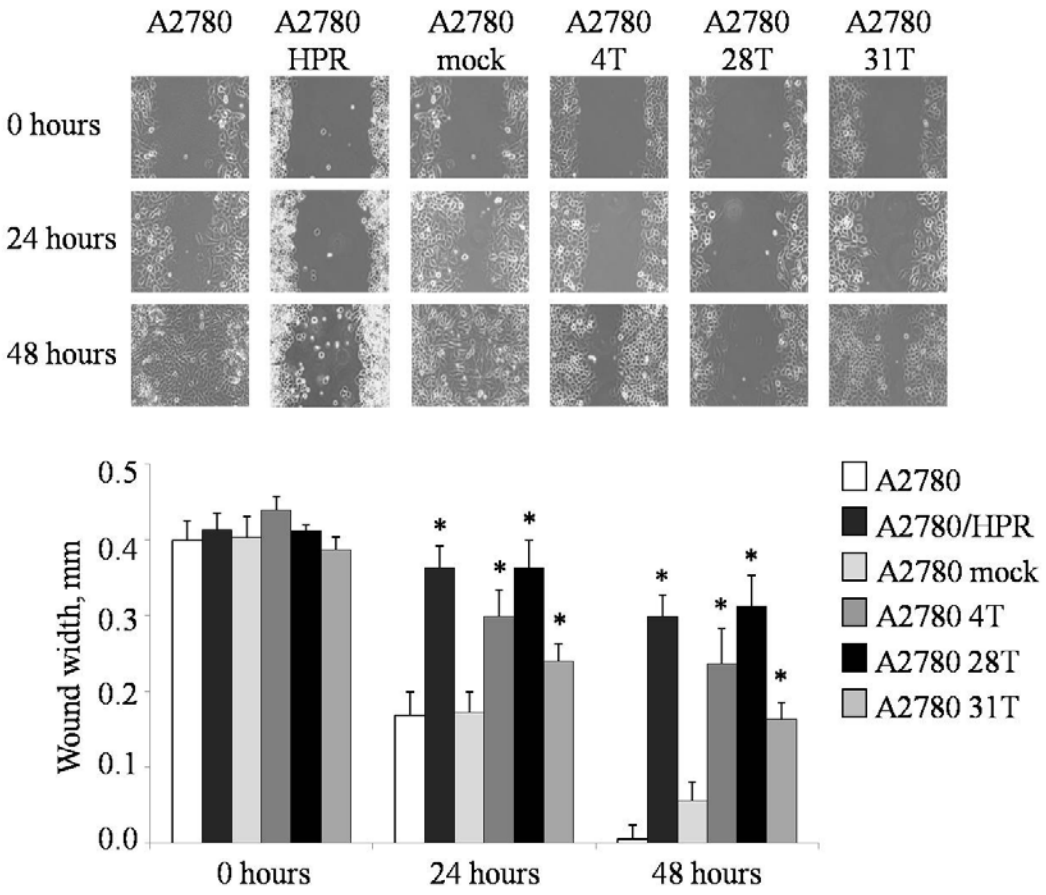


Figure 2. Effect of GM3 synthase (SAT-I) overexpression on the *in vitro* motility of A2780 cells. *In vitro* motility of A2780, A2780/HPR and A2780 cells transfected with the empty expression vector (mock) or the vector containing the SAT-I cDNA (clones 4T, 28T, and 31T) were assessed by the wound healing assay. Confluent monolayers were wounded with a 200-µl pippet tip; phase-contrast microscopy images of the wound (upper panel shows representative images for each data set) have been recorded at different times and the wound width has been measured at 0, 24, and 48 h. Data are expressed in mm and are the means ± S.D. of three different experiments. *, $p < 0.05$ *versus* A2780 cells.

In vitro adhesive ability of A2780, A2780/HPR and A2780/SAT-I cells

The α5β1 integrin has been reported to bind to the extracellular matrix components fibronectin, type I collagen, and laminin (230). The ability of the SAT-I transfected monoclonal cells to adhere to fibronectin, major ligand of α5β1 integrin, was compared with that of A2780 wild-type cells and A2780/HPR cells. Adhesion to bovine serum albumin (BSA) was also examined as a non α5β1 integrin-mediated adhesion control. Adhesion was examined after a 30 min incubation of the cells on the coated wells using a MTT reduction assay as described in Materials and Methods section. After 30 min of incubation, there was nearly no cell binding to BSA coated wells. On the fibronectin coated wells, both SAT-I tansfected 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 result observed previously. GM3 synthase overexpression resulted in an increase of cell adhesion, meanwhile a decrease of cell *in vitro* motility, suggesting a possible role of gangliosides in controlling cell motility and adhesion.

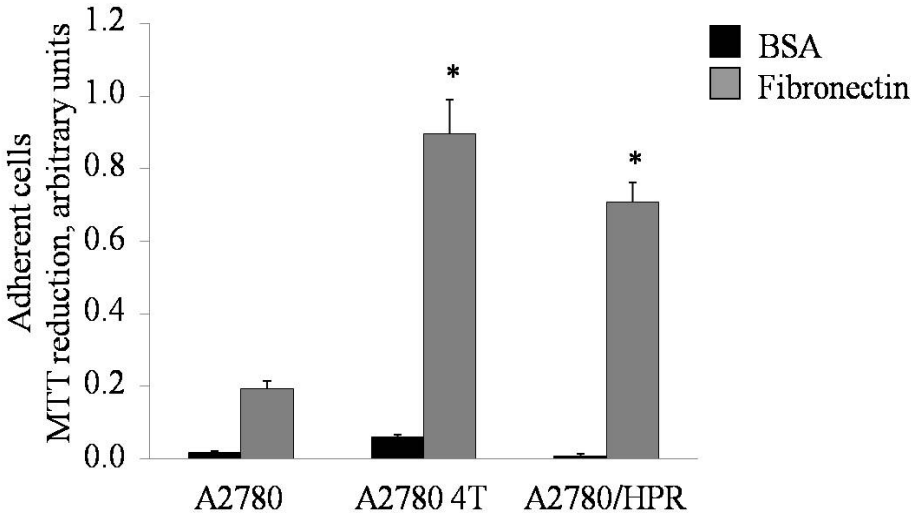


Figure 3. *In vitro* adhesion assay of A2780, A2780/HPR and A2780/SAT-I cells. Human fibronectin and bovine serum albumin

(BSA) were coated in 24-well plate at 4 °C overnight. On the other day, after wash with PBS, 1×10^5 cells were plated in each well and were incubated at 37 °C for 30 min allowing cell adhesion. Then nonadherent 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.

Expression of caveolins in A2780 and A2780/SAT-I-transfected clones

Caveolin-1 has the potential as a molecular organizer for ganglioside-modulated signaling complexes. Caveolin-1, as the main structural component of *caveolae* (89), not only localizes in lipid-enriched membrane domain, but also transmits the signaling from ganglioside formed complexes. In general, caveolin-1 is supposed to concentrate signaling molecules (74,94,119) within specialized membrane domains, but its functional importance is still unclear (6). Caveolin-1 has a phosphorylation site, tyrosine 14, which is supposed to regulate Src activation. Caveolin-1 can also act as a membrane adapter, coupling the integrin subunit to cytosolic Src-family protein tyrosine kinases (174). Since inhibition of integrin-mediated Src signaling by gangliosides negatively regulates tumor cell adhesion and motility, the link between caveolin-1 and glycosphingolipids is worthy to be further investigated.

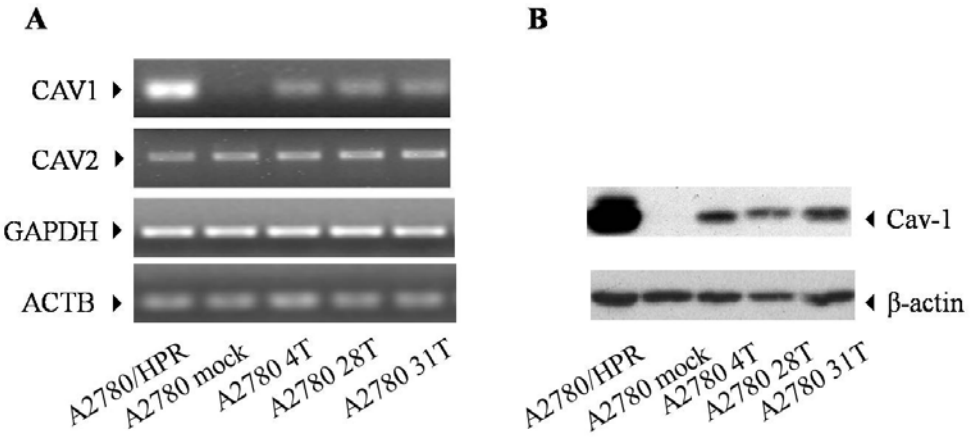


Figure 4. Expression of caveolins in A2780 and A2780/SAT-I-transfected clones. (Panel **A**) mRNA levels of CAV1 and CAV2 were assessed by RT-PCR. GAPDH and ACTB mRNA expressions were measured as an internal control. (Panel **B**) Caveolin-1 protein levels were assessed by Western blotting. Equal amounts of cellular proteins (corresponding to 30 μg) were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed using specific anti-caveolin-1 and anti-β-actin monoclonal antibodies. Patterns are representative of those obtained in three different experiments.

The expression of caveolin-1 in A2780 cells has been determined through semi-quantitative PCR for mRNA levels analysis (Figure 4, Panel A), and through SDS-PAGE followed by Western blotting analysis for the protein analysis (Figure 4, Panel B). Both of caveolin-1 mRNA and protein levels were markedly up-regulated in all SAT-I transfected clones (4T, 28T and 31T) compared with wild type and mock-transfected A2780 cells, in which the protein level of caveolin-1 is very low, in agreement with previous published data (231). This difference was specific within the caveolin gene family for caveolin-1, since caveolin-2 mRNA levels were not affected and caveolin-3 was not detectable in our cells (data not shown).

Since caveolin-1 was upregulated in all the cells characterized by a high expression of the enzyme GM3 synthase (including A2780/HPR cells), we were inclined to explore

the possible connection between the two membrane components: ganglioside and caveolin-1.

Effect of c-Src activity on A2780 cell *in vitro* motility

Since Src kinases can act as a downstream effector of caveolin-1-mediated signaling (174), we tested the effect of Src kinase inhibition on the motility of A2780 cells. The *in vitro* motility of A2780 cells was strongly reduced in the presence of 3 μM SU6656 (a selective Src inhibitor) assessed by wound healing assay (Figure 5, Panel A). The inhibition of Src was confirmed by detecting active and inactive form of Src through Western blotting. As shown in Figure 5 (Panel B), there was only a strong reduction of active form of Src (Src-pY416) in the presence of the inhibitor of Src. However, the total Src expression and inactive form of Src (Src-pY527) were not changed. Thus we may draw the conclusion that Src activity is also involved in modulating A2780 human ovarian carcinoma cell motility. The relationship among Src activity, caveolin-1 and glycosphingolipid is worthy to be further studied.

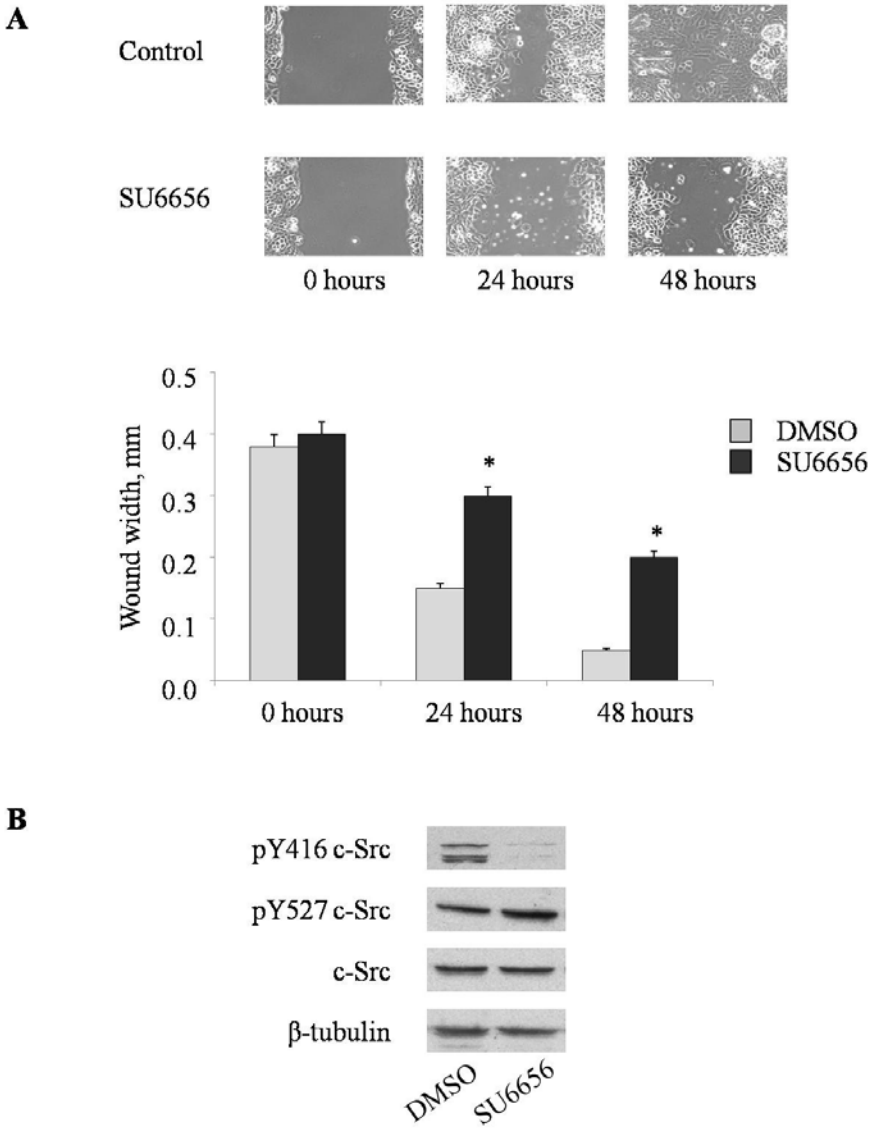


Figure 5. Effect of the c-Src inhibitor on *in vitro* motility of A2780 cells. A2780 cells were incubated in 10% FBS containing medium in the presence of equal volume of dimethyl sulfoxide (DMSO), as a negative control or 3 μM SU6656 Src inhibitor. (Panel **A**) Monolayers were wounded and wound width was measured at 0, 24, and 48 hours, phase-contrast microscopy images of the wounds (upper panel shows representative images for each data set). (Panel **B**) After 48 hours, proteins were exacted for analyzing c-Src activity by probing with specific antibodies against active form of Src (Src-pY416) and inactive form of Src (Src-pY527). Data are expressed in mm and are the means ± S.D. of three different experiments. *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

Effect of exogenous administration of gangliosides on the *in vitro* motility of A2780 cells

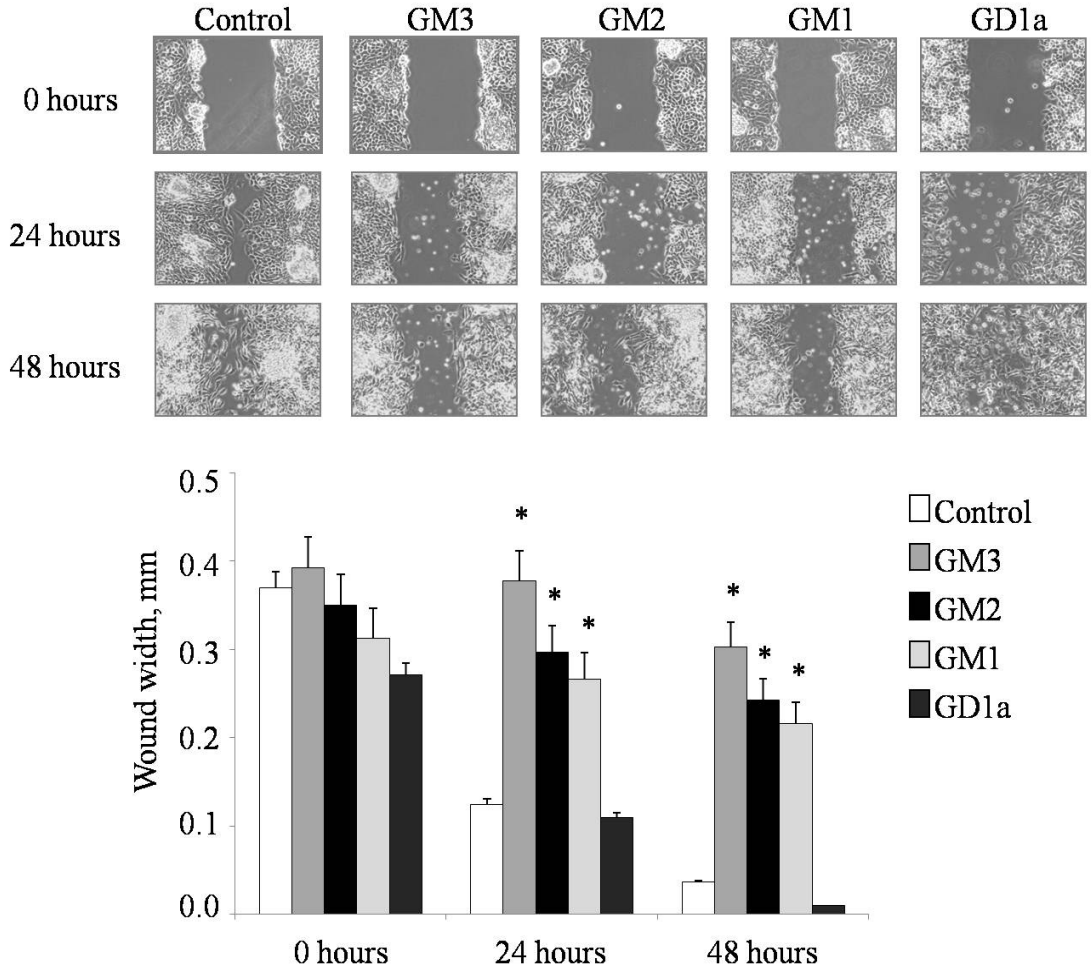


Figure 6. Effect of exogenous administration of gangliosides on *in vitro* motility of A2780 cells. A2780 cell confluent monolayers were wounded and incubated in serum-free medium in the presence of vehicle (Control) or 50 μM of GM3, GM2, GM1, or GD1a for up to 48 h. Phase-contrast microscopy images of the wound (upper panel shows representative images for each data set) have been recorded at different times, and the wound width (lower panel) has been measured at 0, 24, and 48 h. Data are expressed in mm and are the means ± S.D. of three different experiments. **, $p < 0.05$ versus* controls, cells treated with vehicle only.

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 cells in the presence of exogenous gangliosides under experimental conditions that allowed us to increase by 3- to 20-fold the trypsin-stabile cellular content of the administered ganglioside (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. On the other hand, GD1a, present as a minor ganglioside in A2780 cells but representing 15-22% of total gangliosides of SAT-1-transfected clones, had no effect on A2780 cell motility (Figure 6).

Effect of exogenous administration of gangliosides on A2780 cells *in vitro* adhesive ability

As reported above, SAT-I overexpression altered A2780 human ovarian carcinoma cell adhesive ability. If the content of gangliosides related to the cell adhesion was further verified by exogenous administration of gangliosides in A2780 cells. Briefly, A2780 cells were pre-treated with gangliosides for 48 hours as described in Materials and Methods section. Cell adhesion assay was conducted by using fibronectin-coated 24-well plates. As shown in figure 7, there was no significant difference of the tendency of binding to BSA-coated wells among control, ganglioside GM3- and GD1a-treated A2780 cells. However, the cells treated with ganglioside GM3 attached more strongly to the fibronectin coated plated, compared to control and GD1a treated cells. 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, thus, inhibited the cell *in vitro* motility. Notably, fibronectin, as an important

extracellular matrix, has a specific affinity to integrin α5β1, which is the main type of integrin expressed in A2780 cells, suggesting that integrin α5β1 is involved in GM3-mediated A2780 cell *in vitro* motility and adhesion.

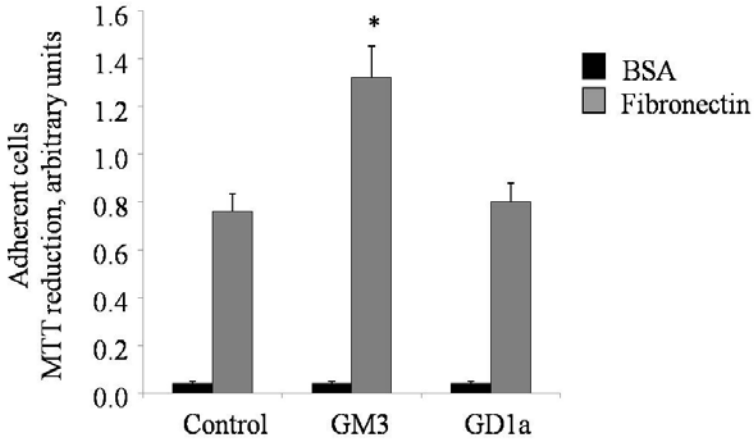


Figure 7. Effect of exogenous administration of gangliosides on *in vitro* adhesion of A2780 cells. A2780 cell confluent monolayers were pre-treated with vehicle (Control) or 50 μM GM3 or GD1a in serum-free condition for 48 hours. After that, cells were counted and were used for evaluating cell adhesive ability following the procedure as described in "Materials and Methods". Data are the means ± S.D. of three different experiments. *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

Effect of PDMP treatment on ganglioside patterns in A2780/HPR and SAT-I cells

High expression levels of GM3 synthase, leading to high cellular gangliosides content, in both A2780/HPR and A2780/SAT-I-transfected cells were associated with a reduction of *in vitro* cell motility and an increase of cell adhesion. Accordingly, 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 of these cells. To confirm this hypothesis, we assessed the effect of the pharmacological manipulation of ganglioside levels on the *in vitro* motility of A2780/HPR and SAT-I cells. Treatment of A2780/HPR and SAT-I cells with the specific GlcCer synthase inhibitor D-PDMP strongly reduced gangliosides content after 2 days (Figure 9) and almost completely abolished GlcCer, LacCer, and ganglioside synthesis after 5 days (Figure 8). L-PDMP (the inefficient isomer, used as a negative control) had no effect on glycosphingolipid levels in A2780/HPR and SAT-I cells. Both of L- and D-PDMP did not alter the expression ceramide and sphingomyelin (SM) expression in A2780/HPR and SAT-I cells. Taken together, we know that the effect of D-PDMP is specific inhibition of the glycosphingolipids synthesized from GlcCer, however, the isomer L-PDMP is not. According to this, PDMP treatment can be an appropriate model to study the role of ganglioside in A2780 cells.

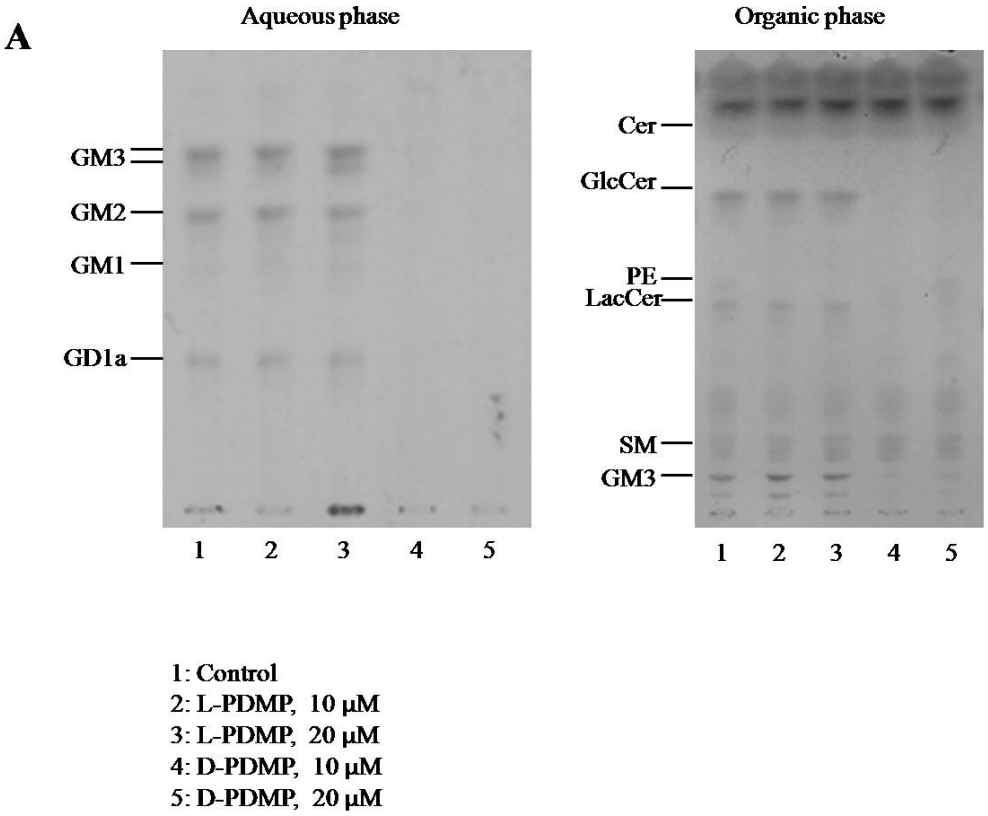


Figure 8. Effects of PDMP treatment on the sphingolipid composition in A2780/HPR cells. A2780/HPR cells were treated with the specific glucosylceramide synthase inhibitor D-PDMP to achieve sphingolipid depletion. The ineffective stereoisomer L-PDMP has been used as a negative control. Here are the sphingolipid patterns of A2780/HPR cells untreated (Control), treated with 10 μM and 20 μM L-PDMP or treated with 10 and 20 μM D-PDMP for 5 days. Cell lipids were extracted with chloroform/methanol/water, 2:1:0.1 by volume, subjected to a two-phase partitioning. Aqueous phases (left panel) and organic phases (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, aniline/diphenylamine) for organic phases as solvent system respectively. The equivalent to 1 mg (for aqueous phases) or 500 μg (for organic phases) of cell proteins were loaded on each lane. SM, sphingomyelin. PE, phosphatidylethanolamine.

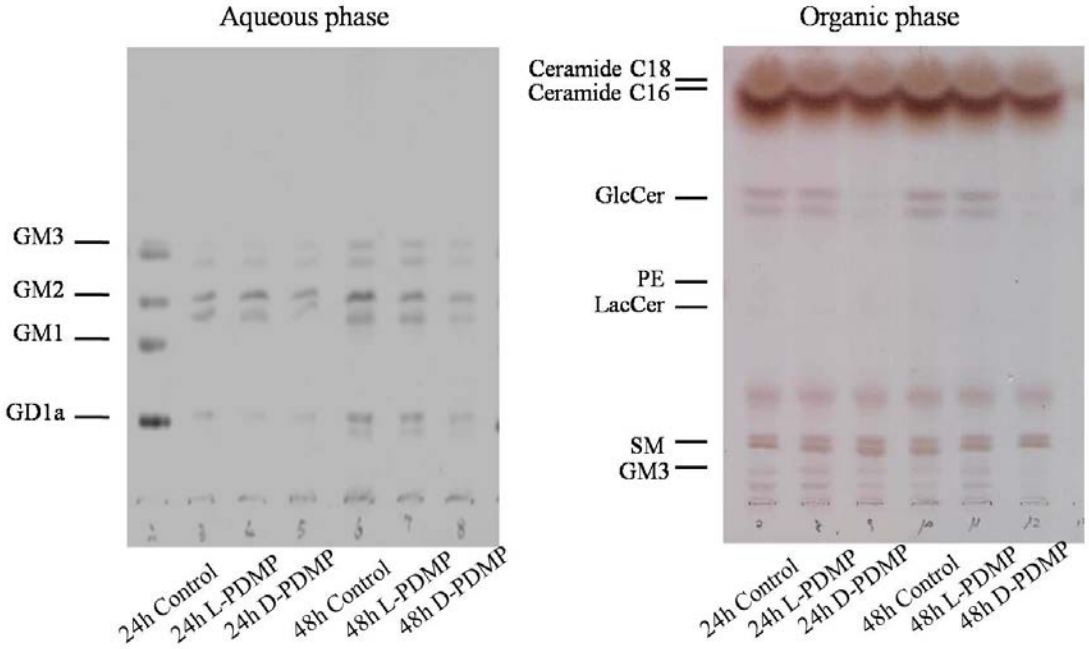


Figure 9. Effects of PDMP treatment on the sphingolipid composition in A2780/SAT-I cells. A2780/SAT-I 4T cells were treated with 20 μM L- or D-PDMP for 24 and 48 hours. Cell lipids were extracted with chloroform/methanol/water, 2:1:0.1 by volume, subjected to a two-phase partitioning. Aqueous phases (left panel) and organic phases (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 (for aqueous phases) or 1 mg (for organic phases) of cell proteins were loaded on each lane.

Effect of PDMP treatment on *in vitro* motility of A2780/HPR and SAT-I cells

Treatment with L- or D-PDMP was not toxic; however, it slightly reduced A2780/HPR 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 on their dorsal surface small particles 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/HPR and SAT-I cells as shown in Figure 10 and 11. Above all, these results further support 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; on the contrary, low content of gangliosides resulted in high cell motility.

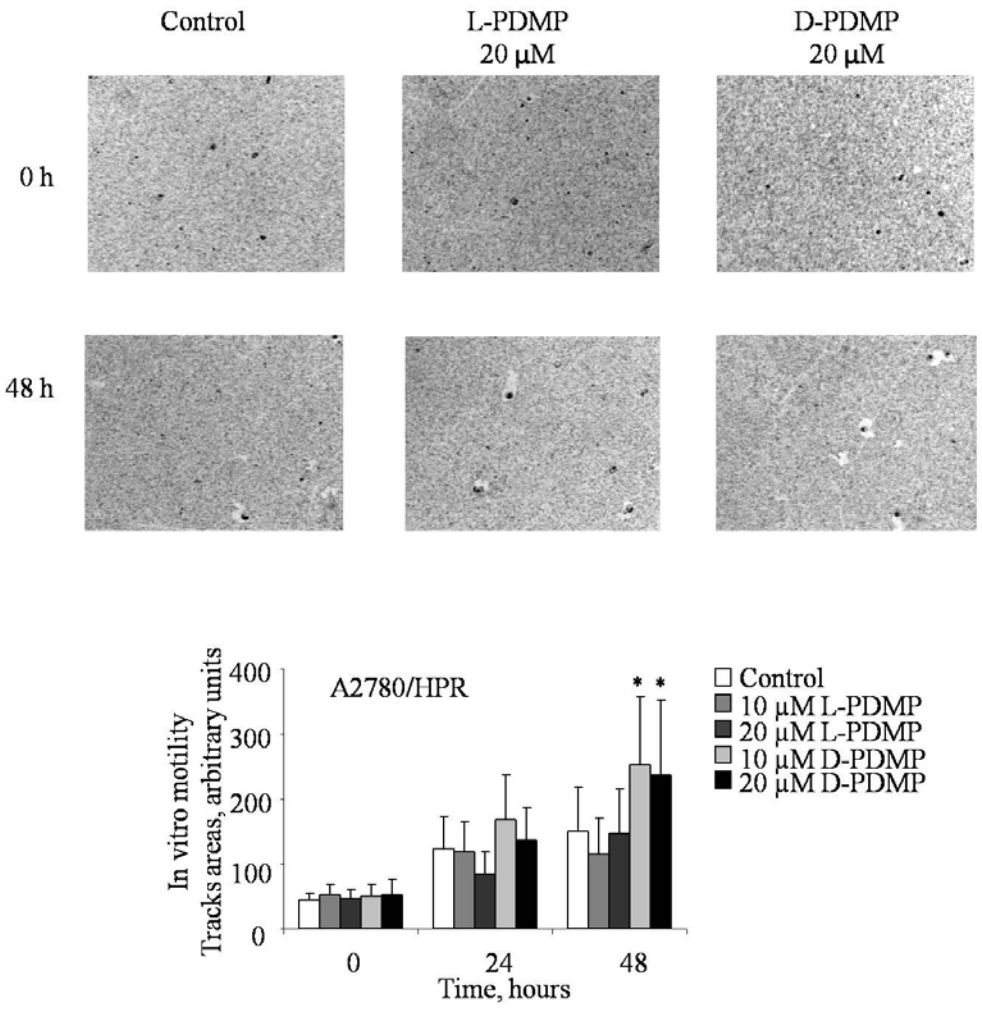


Figure 10. Effect of PDMP treatment on *in vitro* motility of A2780/HPR cells. A2780/HPR cells were treated with 10 or 20 μM of L- or D-PDMP for 48 hours. Phagokinetic gold sol assay (PGSA) was performed as described in "Materials and Methods". L- or D-PDMP was maintained in the medium for the whole duration of the assay. Phase-contrast microscopy images of the areas of the tracks cleared by the cells (upper panel shows representative images for each data set) have been recorded at time 0 and after 24 and 48 hours. Average track areas (means ± S.D. of 50 measurements) normalized for the different cell size are reported in the bar graph (lower panel). *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

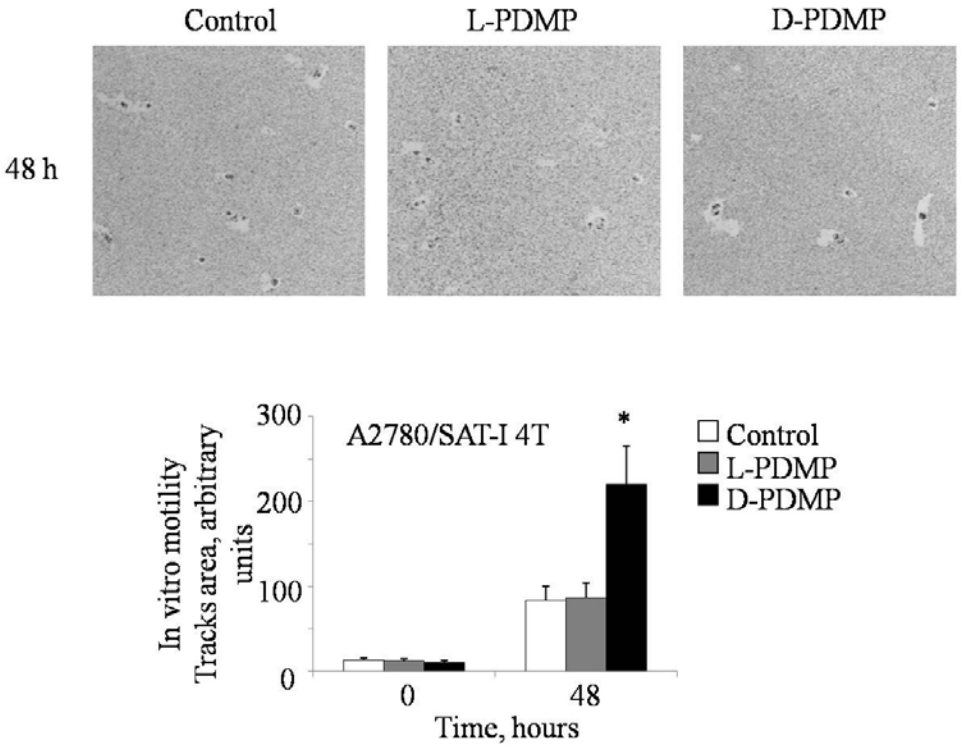


Figure 11. Effect of PDMP treatment on *in vitro* motility of A2780/SAT-I cells. A2780/SAT-I 4T cells were treated with 10 μM of L- or D-PDMP for 48 hours. Phagokinetic gold sol assay (PGSA) was performed as described in "Materials and Methods". The L- or D-PDMP was maintained in the medium for the whole duration of the assay. Phase-contrast microscopy images of the areas of the tracks cleared by the cells (upper 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 (lower panel). *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

Effect of PDMP treatment on A2780/SAT-I cells *in vitro* cell adhesion

Since we have known that PDMP treatment altered the *in vitro* cell motility of A2780/SAT-I cells, and cell motility is tightly correlated with the adhesive ability in this cell. We supposed that PDMP treatment may also affect the cell adhesion. To confirm our assumption, A2780/SAT-I 4T monoclonal cells were pretreated with PDMP for 2 days to inhibit the endogenous gangliosides synthesis. The adhesive ability of these cells was analyzed by following cell adhesion assay as described in Materials and Methods section. As shown in Figure 12, D-PDMP treatment significantly reduced the cell adhesion. However, L-PDMP treatment had no effect on cell adhesion, which was similar as the non treatment control.

Above all, the A2780 cells with high ganglioside GM3 content, including exogenous administration (in A2780 wild type cells), endogenous expression (in A2780/HPR and SAT-I cells), have a high cell adhesion and low motility. This characteristic can be conversed by manipulated depletion of gangliosides levels, indicating that ganglioside GM3 plays an important role in modulating both the cell motility and adhesion in A2780 cells. The mechanism of this regulation and if other molecules also take part in this procedure, need to be further investigated.

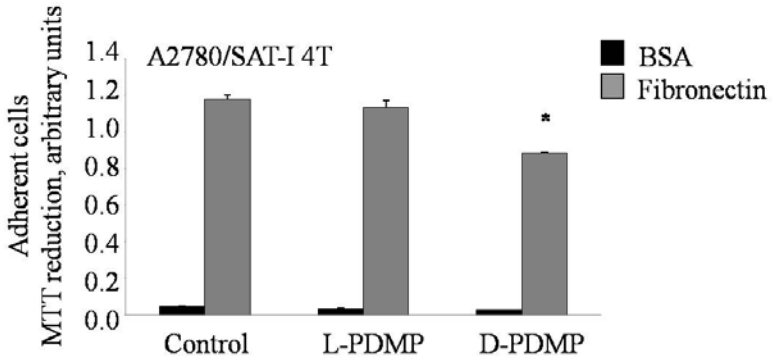


Figure 12. Effect of PDMP treatment on *in vitro* adhesion of A2780/SAT-I cells. A2780/SAT-I 4T cells were pre-treated with 10 μM L- or D-PDMP for 48 hours. After that, cells were counted and were used for evaluating cell adhesive ability following the procedure as described in "Materials and Methods". Data are the means ± S.D. of three different experiments. *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

Transient silencing of caveolin-1 in A2780/HPR and SAT-I cells

As shown above, the caveolin-1 level is different in A2780 cells and the cells that have higher expression level of GM3 synthase, both A2780/HPR cells and SAT-I clones. To further study the correlation between gangliosides and caveolin-1 and their effects on cell motility in our cell model, we silenced caveolin-1 expression from mRNA levels in the A2780/HPR cells and A2780/SAT-I transfected cells using the system of small interference RNA (siRNA) that permits a transient silencing of the target gene. The cells, which were transfected with scrambled siRNA sequences under the same experiment condition, were used as transfection control. The silencing

efficacy was evaluated through western blot analysis and immunofluorescence with the use of a specific antibody. The silencing of siRNA targeting to caveolin-1 gene induced a reduction of about 60 % and 90 % in A2780/HPR and A2780/SAT-I cells for 72 hours transfection, respectively, of the caveolin-1 protein expression respect to control scramble transfected cells (Figure 13, Panel A).

To verify if the reduction in the total expression of caveolin-1 was accompanied by a reduced expression of this protein at the plasma membrane level, an immunofluorescence analysis was performed with specific anti-caveolin-1 antibody in scrambled and CAV1 siRNA transfected A2780/HPR cells. The cells were previously plated on the sterile glass coverslips and the first transient transfection with scrambled or caveolin-1 siRNA was performed after 24 hours and repeated after 48 hours to obtain the maximum possible reduction of caveolin-1 expression. After 48 hours, the cells were fixed, permeabilized and incubated with primary anti-caveolin-1 antibody, followed by incubation with FITC-conjugated secondary antibody. The membrane expression of caveolin-1 in scrambled A2780/HPR cells was clearly visible allowing the determination of cell boundaries, while in the caveolin-1 siRNA transfected cells, the cell membranes were not visible indicating that the caveolin-1 silencing caused a strong reduction in caveolin-1 membrane localization (Figure 13, Panel B). The plasma membrane distribution of phosphorylation site of caveolin-1, Cav-1-pY14, was also detected by this immunofluorescence method (data not shown). Since caveolin-1 is phosphorylated in active regions of the cell membrane, such as focal adhesions, the presence of Cav-1-pY14 is not spread to the entire membrane but limited to a few clusters. For this reason the immunofluorescence of scrambled A2780/HPR cells with a specific antibody of Cav-1-pY14 showed just a few visible spots of cell membrane. In the siRNA transfected A2780/HPR cells these Cav-1-pY14 clusters on the cell membrane were no longer visible and this result was in agreement with the reduction of Cav-1-pY14 expression analysed by western blot (data not shown). These data suggest that the caveolin-1 silencing in A2780 cells is not only

able to reduce the total expression of caveolin-1, but also can reduce the protein localization on the cell membrane and its activation consequent to pY14 phosphorylation.

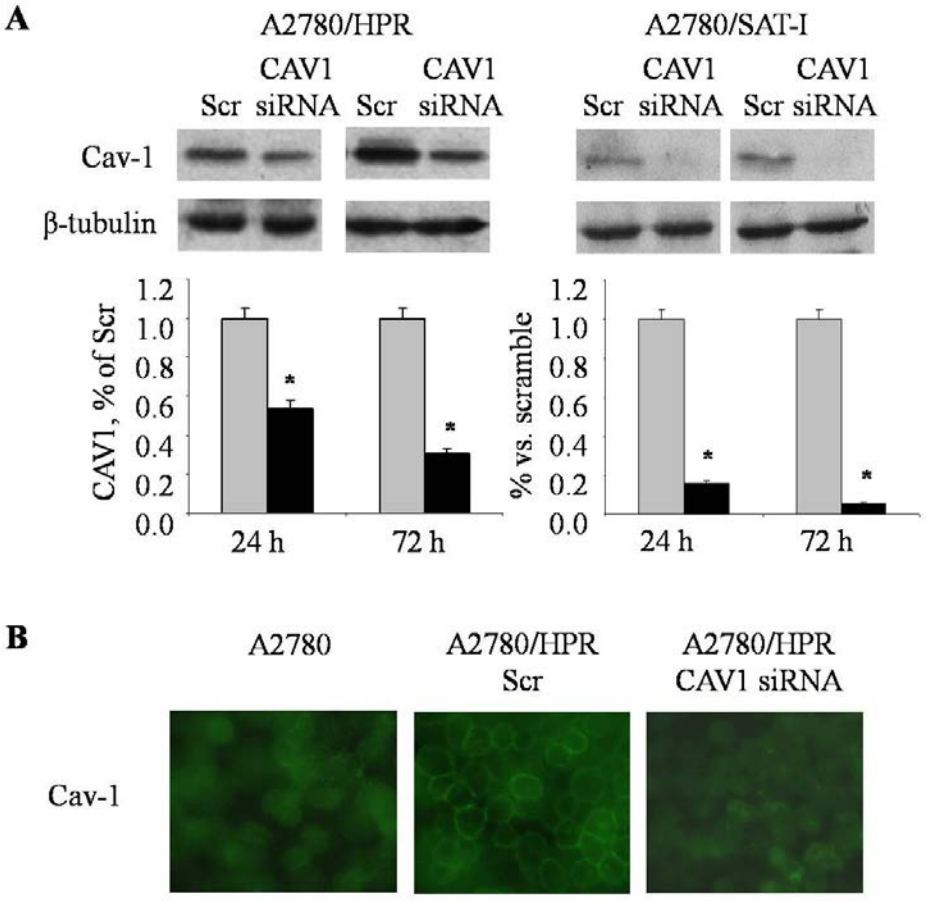


Figure 13. Caveolin-1 silencing in A2780/HPR and A2780/SAT-I-transfected A2780 cells. A2780/HPR and SAT-I-transfected A2780 cells have been transfected with siRNA targeting to CAV1 gene to reduce the expression of caveolin-1. (Panel **A**) Western blot analysis and quantification of caveolin-1 expression in A2780/HPR and SAT-I-transfected A2780 cells transfected with siRNA targeting to CAV1 mRNA (black bars) or with scrambled siRNA as transfection control (Scr) (gray bars). Analysis has been performed 24 hours and 72 hours after siRNA administration. β-tubulin was detected as a loading control. Patterns are representative of those obtained in three independent experiments (upper panel). The amount of caveolin-1 present in each

sample was determined by densitometry, normalized respect to β-tubulin, and expressed as a percentage of time-matched controls. Data are the means ± S.D. of three independent experiments. (Panel **B**) Detection of caveolin-1 protein by immunofluorescence analysis. Untransfected A2780 cells (left) and A2780/HPR cells transfected with scrambled siRNA (middle) or with siRNA targeting to CAV1 (right) were permeabilized, exposed to a rabbit monoclonal anti-caveolin-1 antibody, and then incubated with a secondary anti-rabbit antibody conjugated to FITC as described under "Materials and Methods". Fluorescence images are representative of those obtained in three independent experiments. *, $p < 0.01$ *versus* controls, cells treated with vehicle only.

Effect of caveolin-1 silencing on the proliferation level and *in vitro* cell motility in A2780/HPR and 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 motility, 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 (Figure 14, Panel A).

According to this result, wound healing assay could be used to assess the *in vitro* motility of caveolin-1 silencing in A2780/HPR and A2780/SAT-I cells. To verify this, caveolin-1 was silenced by siRNA transfection as described before, and after 24 hours the wound healing assay was performed and continued for another 48 hours. As shown in Figure 14 (Panel B), the *in vitro* motility of A2780/HPR and SAT-I transfected A2780 cells, assessed by wound healing assay, was markedly higher in caveolin-1 silenced cells compared with scramble sequence transfected cells. A significant difference in cell motility was evident already after 48 hours. Seventy two

hours after the scratch, the caveolin-1 knocked down A2780/HPR and SAT-I transfected cells completely healed the wound, suggesting a leading role of caveolin-1 in the regulation of the cell motility signal in this cell model.

At the end of wound healing assay, the cells were lysed for protein and lipid analysis. The protein expression of caveolin-1 analyzed by Western blotting showed a similar reduction pattern as previous result in caveolin-1 silenced cells (data not shown). The gangliosides compositions analyzed by HPTLC showed that there was no any difference between caveolin-1 silenced cells and scramble sequence transfected A2780 cells (data not shown).

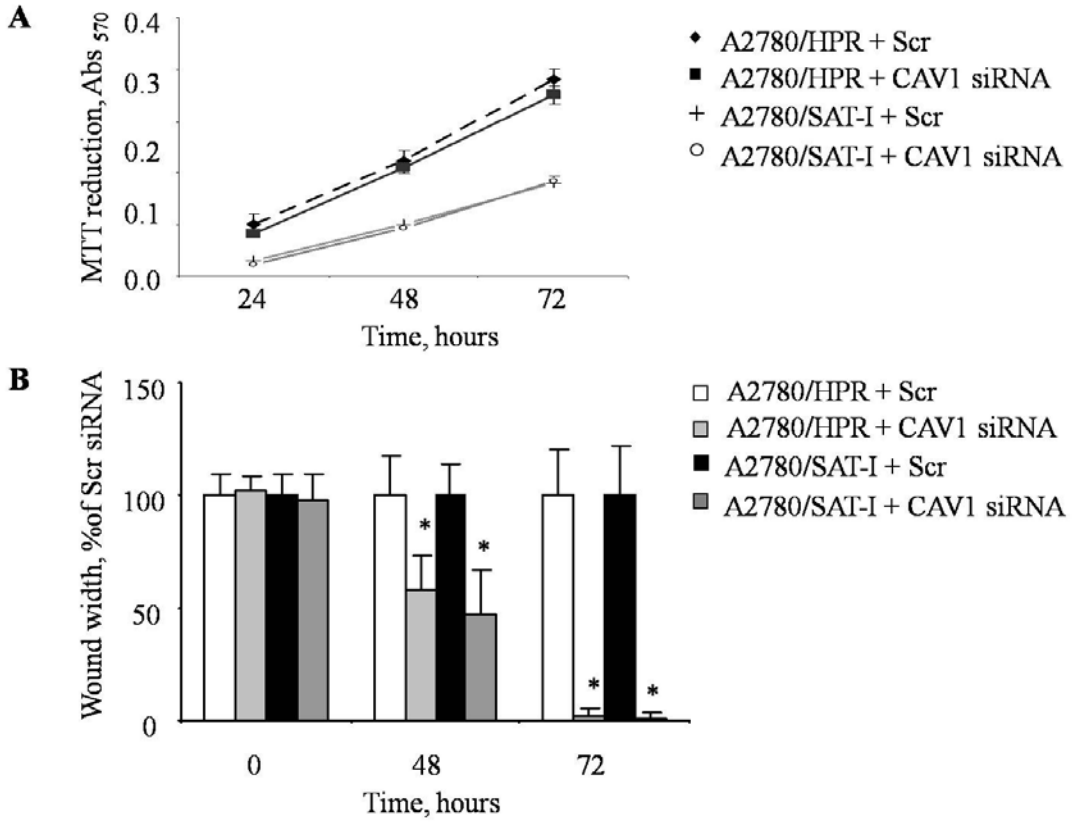


Figure 14. Effects of caveolin-1 silencing on cell proliferation level and *in vitro* cell motility of A2780/HPR and A2780/SAT-I cells. (Panel **A**) Cell proliferation level of A2780/HPR and A2780/SAT-I cells transfected with siRNA for CAV1 gene or with scrambled siRNA was analyzed by MTT reduction assay measured

after 24, 48 and 72 hours plated cells. (Panel **B**) Wound healing assay was performed on the cited cells, as described in Materials and Methods section. Phase-contrast images of the wounds were recorded at different times and the wound widths were measured. Data are expressed in percentage respect to cells treated with scrambled sequence and are the means ± S.D. of three different experiments. *, $p < 0.001$ *versus* controls.

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

Since silencing of caveolin-1 by siRNA influenced the existence of pY14 Cav-1 on the cell plasma membrane as shown above, 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* (176). 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 in A2780 cells. It confirmed again that caveolin-1 participated in gangliosides modulating cell signaling, and it suggested that gangliosides mediated A2780 cell motility through caveolin-1 phosphorylation.

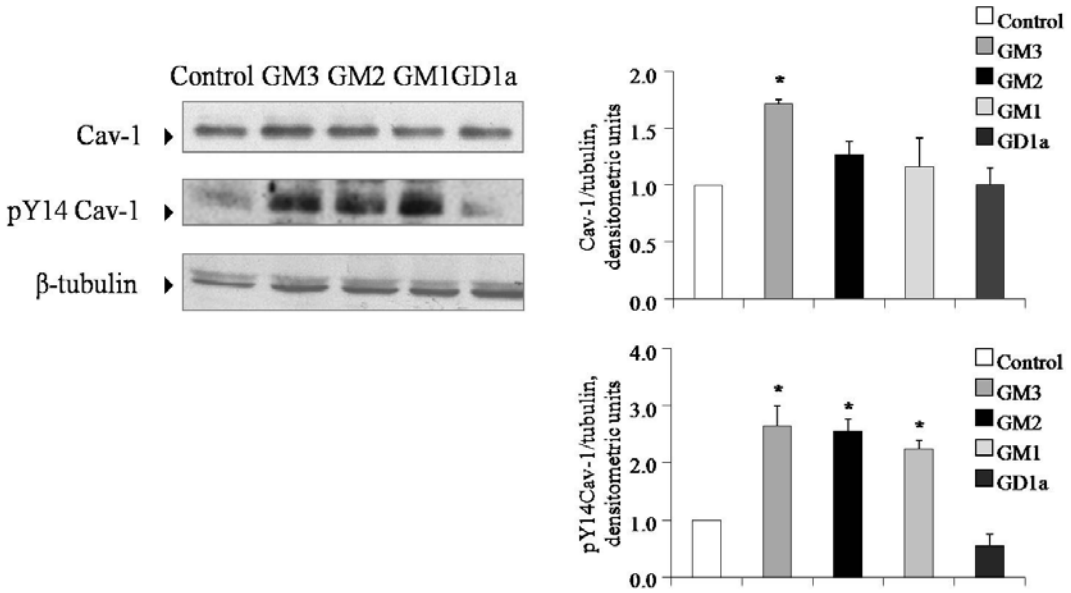


Figure 15. Effects of exogenous administration of gangliosides on caveolin-1 expression and caveolin-1 phosphorylation level. A2780 cell confluent monolayers were administrated with vehicle (Control) or 50 μM of GM3, GM2, GM1, or GD1a for up to 48 h. After that, cells were analyzed by Western blotting 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 independent 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, A2780/SAT-I 4T cells were treated with L- or D-PDMP for 24 hours or 48 hours. After the treatment, cells were lysed to analyze by Western blotting with specific antibodies against caveolin-1 and pY14 Cav-1. As shown in Figure 16, 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.

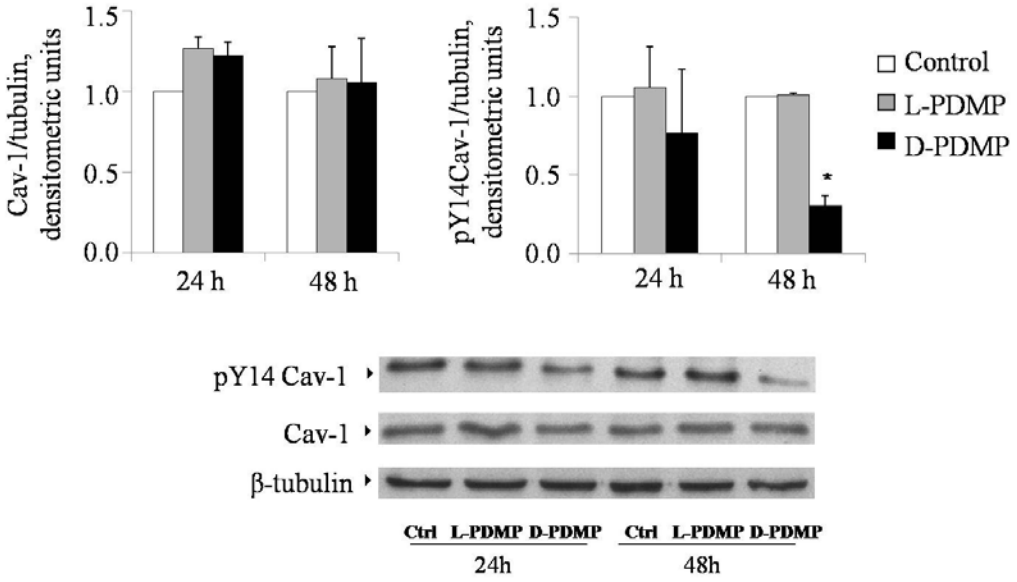


Figure 16. Effects of PDMP treatment on caveolin-1 expression and caveolin-1 phosphorylation level. A2780/SAT-I 4T cells were treated with 20 μM L- or D-PDMP for 24 and 48 hours. Total cell lysate from control or PDMP treatment cells were analyzed by Western blotting detection using specific antibodies 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 (uppper panel). Data are the means ± S.D. of three independent experiments. *, $p < 0.01$ *versus* controls, cells treated with vehicle only.

Effects of the modulation of caveolin-1 and cellular ganglioside levels on c-Src activity

These results indicate that both gangliosides and caveolin-1 exert an inhibitory regulation on the motility of A2780 human ovarian carcinoma cells. In addition, they suggest that gangliosides and caveolin-1 might be organized in a multimolecular complex together with c-Src and integrin receptors. Several papers indicated that GM3 is able to negatively affect cell motility stabilizing the formation of a integrin/tetraspanin membrane complex, leading to the inhibition of c-Src (81,86,232,233). On the other hand, caveolin-1 suppression of metastatic potential in osteosarcoma (234) and melanoma (235) has been linked to the inhibition of c-Src activation. In addition, we have previously shown that the motility of low GM3 synthase-expressing A2780 cells was reduced in the presence of a Src inhibitor, and that c-Src was less active in A2780 SAT-I-transfected cells (4). To confirm the role of c-Src in the control of cell motility in this cell model, we assessed Src kinase activity after different treatments were able to modify the gangliosides or caveolin-1 levels in these cells, resulting in variations in the cell *in vitro* motility.

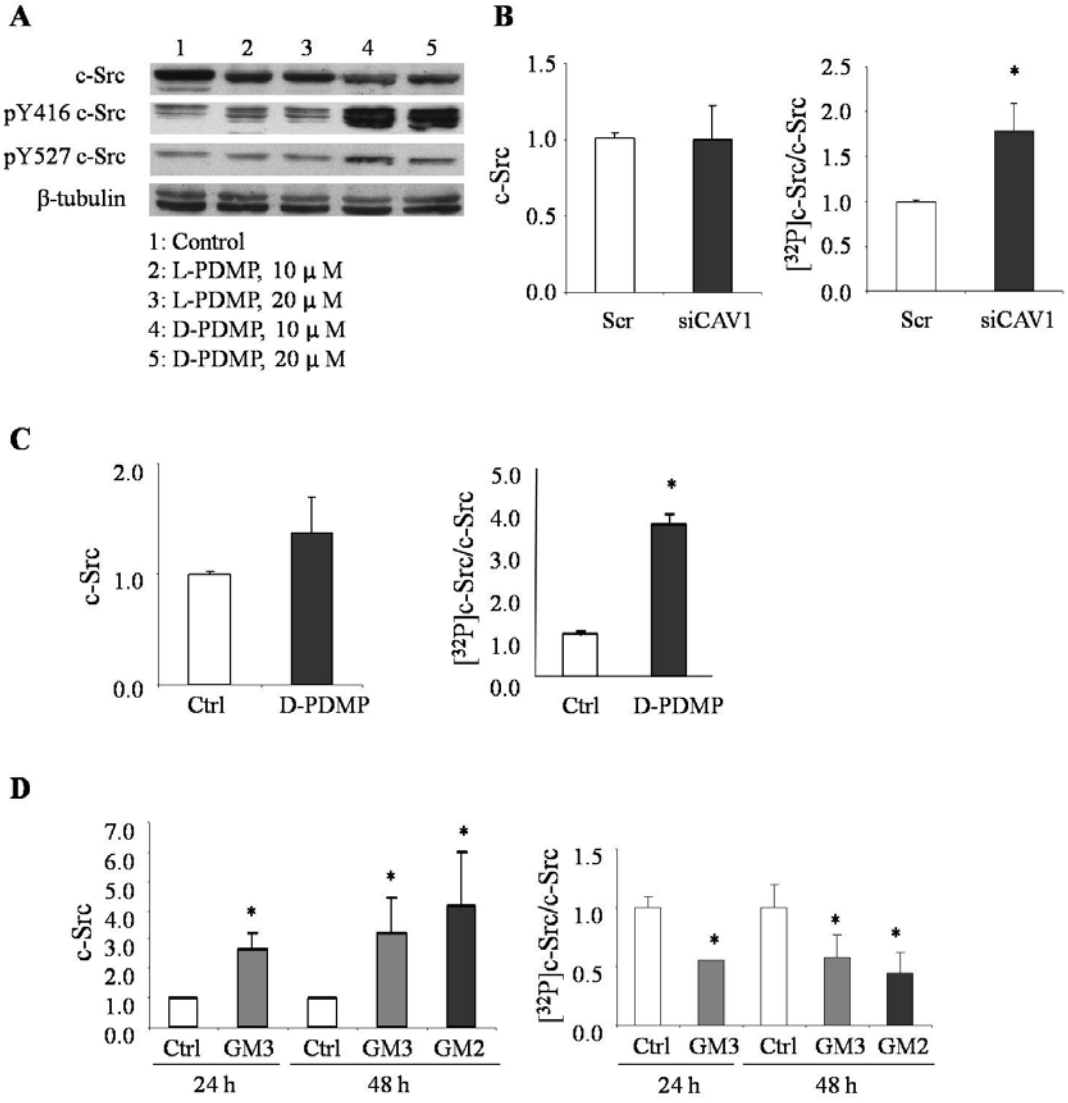

A
1 2 3 4 5
c-Src
pY416 c-Src
pY527 c-Src
β-tubulin
1: Control
2: L-PDMP, 10 μ M
3: L-PDMP, 20 μ M
4: D-PDMP, 10 μ M
5: D-PDMP, 20 μ M
B
c-Src
Scr
siCAV1
[32P]c-Src/c-Src
Scr
siCAV1
C
c-Src
Ctrl
D-PDMP
[32P]c-Src/c-Src
Ctrl
D-PDMP
D
c-Src
Ctrl GM3 Ctrl GM3 GM2
24 h
48 h
[32P]c-Src/c-Src
Ctrl GM3 Ctrl GM3 GM2
24 h
48 h

Figure 17. Effects of the modulation of caveolin-1 and cellular ganglioside levels on c-Src activity. (Panel **A**) A2780/HPR cells were treated with 10, 20 μM L- or D-PDMP for 48 hours. Total cell lysate from control or PDMP treatment cells were analyzed by Western blotting detection using specific antibodies against c-Src, c-Src-pY416 and c-Src-pY527. β-tubulin was simultaneously detected as a loading control. Patterns are representative of those obtained in three independent experiments. (Panel **B**-**D**) c-Src activity has been measured as autophosphorylation using an immunocomplex kinase assay as described under "Materials and Methods". Briefly, c-Src has been immunoprecipitated with a specific monoclonal antibody from cell lysates obtained under the different experimental conditions. Immunoprecipitates have been incubated with 10 μM [γ-^{32}P]ATP. After incubation, proteins associated with each sample have been separated by SDS-PAGE. The total amount of c-Src associated with each sample has been determined by Western blotting and densitometry (left panels). The amount of radioactivity associated with the band corresponding to c-Src protein has been determined by autoradiography and has been normalized for the total c-Src content associated with each sample (right panels). (Panel **B**) Effect of caveolin-1 silencing in A2780/HPR cells. A2780/HPR cells were transfected with siRNA targeting to CAV1 mRNA (black bars) or with scrambled siRNA as control (white bars). Analysis has been performed 48 hours after siRNA transfection. (Panel **C**) Effect of gangliosides depletion by treatment with D-PDMP in A2780/HPR cells. A2780/HPR cells were treated with 20 μM D-PDMP (balck bars) or with vehicle (white bars) for 48 h. (Panel **D**) Effect of exogenous gangliosides administration in A2780 cells. Cells were treated with 50 μM of GM2 for 48 h or with 50 μM of GM3 for 24 and 48 h in serum-free medium as described under "Materials and Methods". Data are the means ± S.D. of four independent experiments. *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

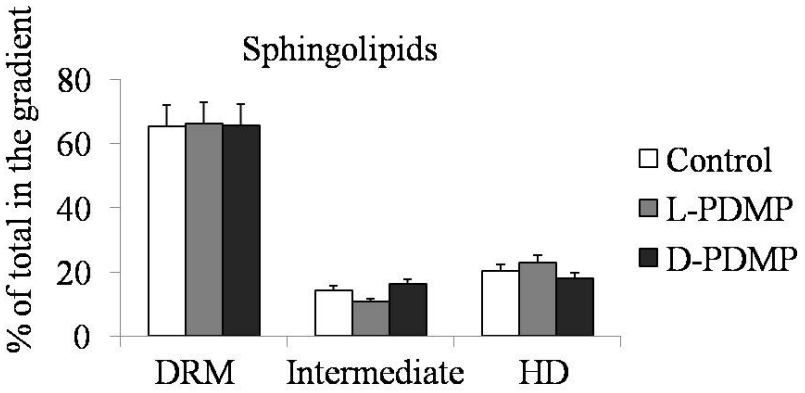
We first checked c-Src and its phosphorylation sites expression under the treatment of L- or D-PDMP in A2780/HPR cells through Western blotting analysis. As shown in Figure 17 (Panel A), the levels of both c-Src and the inactive form of Src (pY527 c-Src) were not changed by L- or D-PDMP treatment. However, the expression of the active form of Src (pY416 c-Src) was strongly elevated in the case of D-PDMP treatment, indicating that c-Src turned to be an active form in the absence of gangliosides.

This result is further confirmed by immunocomplex kinase assay, which is specific useful for detecting tyrosine protein kinase activity. In A2780/HPR cells, abolition of gangliosides synthesis by D-PDMP treatment (associated with increased cell motility) led as well to c-Src kinase activation with similar levels of c-Src protein (Figure 17, Panel C). However, the exogenous administration of monosialoganliosides GM3 and GM2 to A2780 wild type cells (a treatment able to strongly inhibit the cells *in vitro* motility) was able to significantly inhibit c-Src autophosphorylation, even if in this case we observed an increased amount of c-Src protein in the ganglioside treated samples, which might represent a compensatory mechanism (Figure 17, Panel D). Transient silencing of caveolin-1 in A2780/HPR cells (that positively affected *in vitro* cell motility) resulted in a higher c-Src kinase activity with respect to control cells transfected with scrambled sequence, not affecting the total cellular levels of c-Src (Figure 17, Panel B). These data strongly suggest that the activity of c-Src, which is inversely correlated with cell motility, is regulated by the membrane gangliosides composition and/or the presence of caveolin-1.

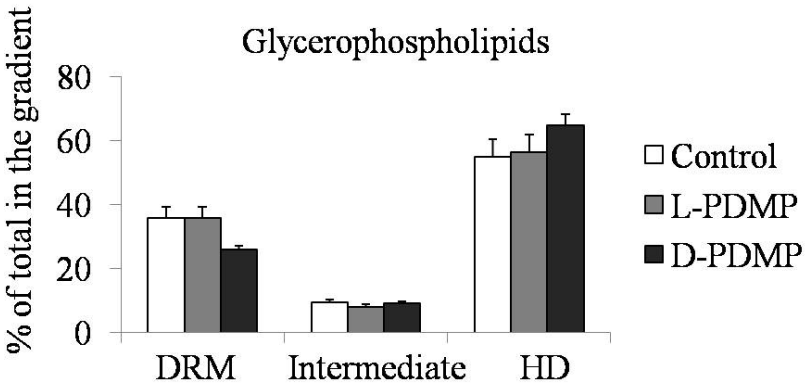
Effects of PDMP treatment on lipids gradient distribution in A2780/SAT-I cells

We have known that D-PDMP treatment inhibited the gangliosides synthesis in A2780/SAT-I cells, in order to analyze the effect of the inhibitor on the gangliosides localized in cell membrane; 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 experimental condition. 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 section. To validate the experimental conditions used for the fractionation, the distribution of sphingolipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS) along the gradient fractions were evaluated after lipid metabolic labeling with [1-^{3}H]sphingosine. As expected, the low density detergent resistant membrane fraction (fractions 4, 5 and 6) was highly enriched in sphingolipids (ceramide, GlcCer, sphingomyelin (SM) and gangliosides), and relatively depleted of glycerophospholipids. The major amount of glycerophospholipids (phosphatidylethanolamine (PE) and phosphatidylserine (PS)) existed in high density fraction (fractions 9, 10 and 11). The fractions 7 and 8 were put together as the intermediate fraction. D-PDMP treatment only slightly reduced the distribution of glycerophospholipids in DRM fraction, however, did not change sphingolipids and other glycerophospholipids distribution in this case (Figure 18, Panel A and B). Cholesterol, another important membrane lipid, which is supposed to bind with caveolin-1 in DRM fraction, was also analyzed by HPTLC. As shown in Figure 18 (Panel C), there was a significant shift of cholesterol from both DRM fraction and intermediate fraction to high density fraction. However, D-PDMP did not alter the total levels of cholesterol in the cells (data not shown). This movement may be caused by the dynamic of caveolin-1 (Figure 19, Panel A); however, the mechanism is needed to be explored.

A


Sphingolipids
% of total in the gradient
80
60
40
20
0
DRM
Intermediate
HD
Control
L-PDMP
D-PDMP

B


Glycerophospholipids
% of total in the gradient
80
60
40
20
0
DRM
Intermediate
HD
Control
L-PDMP
D-PDMP

C

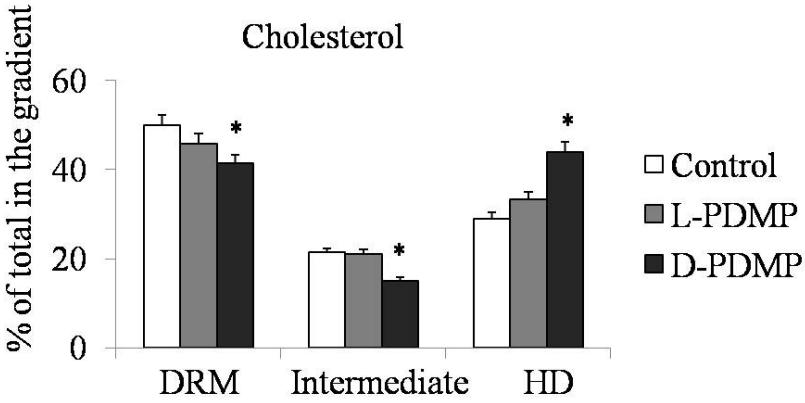

Cholesterol
% of total in the gradient
60
40
20
0
*
*
*
DRM
Intermediate
HD
Control
L-PDMP
D-PDMP

Figure 18. Effects of PDMP treatment on lipids gradient distribution in A2780/SAT-I cells. After metabolic labeling of A2780/SAT-I 4T cell lipids with [1-^{3}H]sphingosine, 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 relative quantities of each lipid 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. (Panel **A-B**) The lipids of different fractions were extracted with chloroform/methanol, 2:1 by volume. The radioactive lipids were separated by HPTLC, using chloroform/methanol/water 55:20:3 by volume. After that, the plate was acquired under Beta-Imager 2000 instrument for at least 3 days. The radioactivity image was quantified with the specific β-Vision software provided by Biospace. The detectable glycerophospholipids in these samples are phosphatidylethanolamine (PE) and phosphatidylserine (PS). The detectable sphingolipids include ceramide, GlcCer, sphingomyelin (SM) and gangliosides. (Panel **C**) The lipids of different fractions were also subjected to a two-phase partitioning and were divided into aqueous phase and organic phase. Cholesterol exists in the organic phase. After methanolyzation to get rid of the glycerophospholipids, the organic phases were analyzed by HPTLC, using hexane/ethyl acetate 3:2 by volume (spray reagent, anisaldehyde). Data are the means ± S.D. of three independent experiments. *, $p < 0.05$ *versus* controls, cells treated with vehicle only.

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

Since PDMP treatment also influenced the caveolin-1 phosphorylation levels, altered Src activity, and changed the integrin-mediated cell adhesion, we are interested to know these protein gradient distributions in the treatment of PDMP. The distributions of proteins usually regard as positive (caveolin-1) or negative (integrin α5) lipid raft markers along the gradient fractions were analyzed by immunoblotting using specific antibodies. As shown in Figure 19, the DRM fraction prepared from SAT-I transfected cells was highly enriched in caveolin-1, whereas integrin α5 was largely recovered in the high density fraction of the gradient.

Under the treatment of D-PDMP, but not L-PDMP, was able to concentrate caveolin-1 to intermediate fraction from both DRM fraction and high density fraction, and this difference was significant (Panel A). There was also a shift of c-Src from DRM fraction to intermediate fraction, however, the distribution did not change in the high density fraction after PDMP treatment (Panel C).

As mentioned above, integrin α5 mediated A2780 cells attachment to fibronectin. Furthermore, integrin α5 usually binds together with gangliosides that forms a gangliosides/integrin α5 complex to regulate cell signals. After D-PDMP treatment, integrin α5 moved out from high density fraction, which existed most of the proteins (about 90%) of the cells, to the DRM fraction and intermediate fraction (Panel B).

Although the distributions of caveolin-1, c-Src and integrin α5 were altered in different patterns, the total expressions of these proteins were not changed by PDMP treatment, suggesting that gangliosides-mediated cell signals via these molecules, not only change the phosphorylation level and kinase activity, but also through changing the distribution of these molecules in the DRM fractions. The mechanism of this regulation and the composition of this signaling complex are worthy to be investigated.

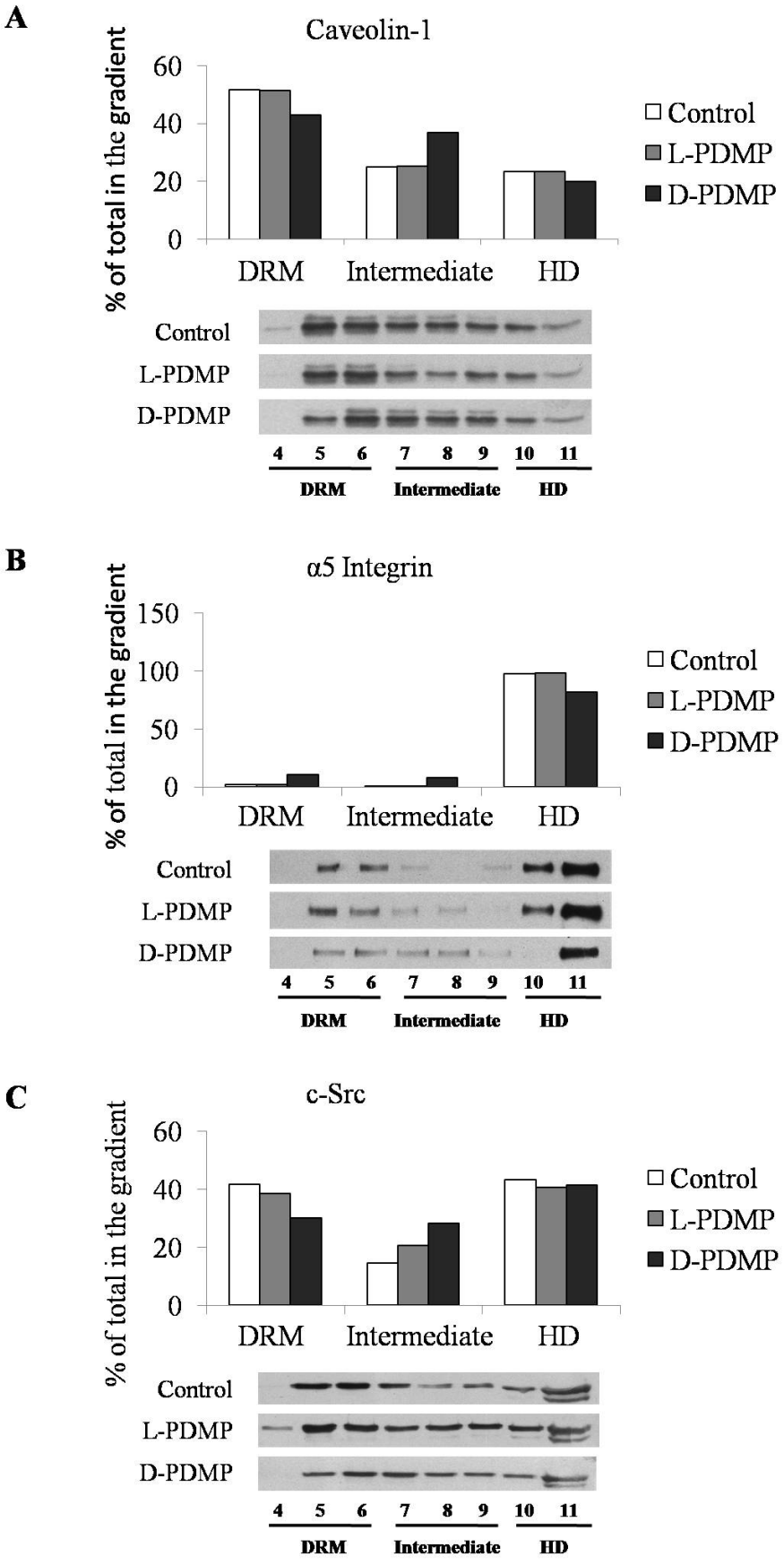

A
Caveolin-1
% of total in the gradient
60
40
20
0
DRM
Intermediate
HD
Control
L-PDMP
D-PDMP
Control
L-PDMP
D-PDMP
4 5 6 7 8 9 10 11
DRM
Intermediate
HD
B
α5 Integrin
% of total in the gradient
150
100
50
0
DRM
Intermediate
HD
Control
L-PDMP
D-PDMP
Control
L-PDMP
D-PDMP
4 5 6 7 8 9 10 11
DRM
Intermediate
HD
C
c-Src
% of total in the gradient
60
40
20
0
DRM
Intermediate
HD
Control
L-PDMP
D-PDMP
Control
L-PDMP
D-PDMP
4 5 6 7 8 9 10 11
DRM
Intermediate
HD

Figure 19. Effects of PDMP treatment on protein gradient distribution in A2780/SAT-I cells. After metabolic labeling of A2780/SAT-I 4T cell lipids with [1-^{3}H]sphingosine, 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 gradient fractions were mixed with 5×sample buffer by the ratio 4:1. The protein distribution was determined by Western blotting, using specific antibodies against caveolin-1, integrin α5 and c-Src. 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. (Panel **A** and **C**) Caveolin-1 and c-Src distribution were detected by Western blotting. The loading volume is 1/100 of the total volume of each fraction. (Panel **B**) For assessing integrin α5 distribution, the loading volume is 1/50 of the total volume from fraction 4 to 9, the loading volume is 1/100 of the total volume for fraction 10 and the loading volume is 1/1500 of the total volume for fraction 11. Patterns are representative of those obtained in three independent experiments.

DISCUSSION

The pieces of evidence provided in my thesis, together with our previously published results (229), indicate that, in human ovarian cancer cells, an increased cellular gangliosides content, caused by the enhanced expression of GM3 synthase, is paralleled by a marked up-regulation of the membrane adaptor protein caveolin-1. The result of concomitantly high levels of gangliosides and caveolin-1 is a marked reduction of *in vitro* cell motility. Up-regulation of caveolin-1 and reduced cell motility were observed in two cellular models in which higher GM3 synthase activity with respect to wild type cells has been obtained by two entirely different procedures: the genetic manipulation of GM3 synthase levels by its stable overexpression in the case of SAT-I-transfected A2780 cells, or the selective pressure in the presence of a drug, *N*-(4-hydroxyphenyl)retinamide) in the case of A2780/HPR cells. The existence of a causal connection between high levels of gangliosides and caveolin-1 and the observed reduction of cell motility is supported by the depletion experiments, as shown in Figure 10, Figure 11 and Figure 14. Both caveolin-1 silencing and block of gangliosides biosynthesis by pharmacological inhibition of glucosylceramide synthase led to a strong enhancement of cell motility. These results indicate that high levels of caveolin-1 and high levels of gangliosides are necessary, but not sufficient, if independent, to down-regulate cell motility. Thus, the regulation of cell motility requires a certain degree of cooperation between gangliosides and caveolin-1. Caveolin-1, an integral membrane protein originally discovered as a main structural component of *caveolae*, soon gained a role as a molecular organizer for multiprotein signaling complexes, due to its 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. Caveolin-1 is insoluble in cold non-ionic detergents (236), and can be enriched in low density, Triton X-100-insoluble membrane fractions. These fractions, highly enriched also 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 and the hydrophobic moiety of the lipid (217,237). 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 as well reported that detergent-resistant association of caveolin-1 and sphingolipids is strong enough to allow co-immunoprecipitation. Caveolin-1 can be immunoprecipitated by a monoclonal antibody to ganglioside GD3 in CHO cells transiently transfected by GD3 synthase cDNA (238).

Here we have 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 (239). Therefore, we hypothesize 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. We tried to elucidate the downstream signaling pathway possibly affected by the ganglioside/caveolin-1 complex. Our results indicated that integrin receptor subunits and the non-receptor tyrosine kinase c-Src are cooperated together with caveolin-1 and sphingolipids within detergent-resistant membrane fractions. Moreover, integrin receptor subunits and c-Src co-immunoprecipitate with caveolin-1. Caveolin-1 (6,7) and Src kinases (172,173) are typically associated with sphingolipid-enriched membrane domains or other subtypes of lipid rafts also in other cell types. In addition, it has been suggested that caveolin-1 might act as a membrane adapter coupling integrin receptors to Src kinases (174), and that caveolin-1-mediated inactivation of the integrin/Src/FAK pathway might be responsible for the inhibition of metastatic potential in melanoma (235). Our results strongly support the hypothesis that the inactivation of c-Src by a 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 cells (expressing low GM3 synthase and low caveolin-1), and that c-Src was less active in SAT-I-transfected cells

(4), characterized by high caveolin-1 levels. Now we report that treatments able to reduce the content of caveolin-1 (CAV1 siRNA) or of gangliosides (glucosylceramide synthase inhibition) in A2780/HPR and in SAT-I-transfected A2780 cells, led to enhanced c-Src kinase activation and increased motility. On the other hand, ganglioside administration to A2780 cells, able to reduce their motility, resulted in c-Src kinase inactivation.

Moreover, we noticed that changing of gangliosides content by D-PDMP treatment did not influence the expression of caveolin-1, c-Src and integrin, but altered the distribution of these molecules. Caveolin-1 moved to the intermediate fraction, which the composition is still unclear so far. Meanwhile c-Src showed the similar pattern as caveolin-1. Interestingly, integrin α5 (also for β1 subunit) (data not shown) shifted from high density to both DRM and intermediate fractions. All of these results imply that the changing of gangliosides compositions resulted in the shift of molecules related to gangliosides signaling. D-PDMP treatment can also reduce caveolin-1 motility (data not shown), thus we supposed that caveolin-1 may bind to other molecules in the case of PDMP treatment. Since both caveolin-1 and integrin α5 inclined to move the intermediate fraction under this condition, integrin α5 is supposed to be a probably candidate associated with caveolin-1 and slows down the motility of caveolin-1 after D-PDMP treatment.

Taken together, all these data suggest a novel role of gangliosides in the regulation of cell motility in human ovarian carcinoma cells, by affecting the organization of a signaling complex organized by caveolin-1 and integrin α5, responsible for Src inactivation.

Our results call the attention to two 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 SAT-I-transfected A2780 cells, as the wild-type A2780 cells. This finding might seem in contrast with the usual association between caveolin-1 and *caveolae*, but is indeed in agreement with the diverse and multiple *caveolae*-independent roles of caveolin-1 that have been described in the last few years (6,7,240). The second aspect is related to the

observation that cellular ganglioside or GM3 synthase levels can regulate the expression of caveolin-1. Yamagata *et al.* showed that GD1a ganglioside regulated caveolin-1 expression in FBJ mouse osteosarcoma cells (241). Also in that case, the concomitant increase in a certain ganglioside 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 high caveolin-1 expression, while the high motility variants contain low ganglioside and low caveolin-1 levels. Treatment of the highly motile FBJ-LL osteosarcoma cell line with exogenous GD1a ganglioside or transfection with GM2/GD2 synthase cDNA resulted in the upregulation of caveolin-1 expression with reduced metastatic potential and suppressed cell adhesion to vitronectin (242). Similarly, GM3 synthase-transfected A2780 ovarian carcinoma cells were characterized by an increased expression of caveolin-1 and reduced *in vitro* cell motility. However, the mechanism underlying the regulation of caveolin-1 expression by the cellular gangliosides levels is totally unknown. The relationship between gangliosides and other molecules related to this signaling pathway need to be further investigated.

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