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XXV CICLE

Isolation and differentiation of stem cells: searching for new markers

Ph.D STUDENT: **Sonia Bergante**

TUTORS: Prof. Bruno Venerando, Prof. Luigi Anastasia

COORDINATOR: Prof. Francesco Bonomi

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ABBREVIATIONS

ES – embryonic stem cells

HSCs- hematopoietic stem cells

UC- umbilical cord

UCB- umbilical cord blood

MSCs- mesenchymal stromal cells

VSELs- very small embryonic-like stem cells

NCSs- neural stem cells

FGF- fibroblast growth factor

EGF- epidermal growth factor

PDGF- platelets-derived growth factor

IQ-1- calmodulin binding motif

CBFA- core binding factor subunit alpha-1

RUNX2- runt related transcription factor 2

HDAC- histone deacetylase

FSK- forskolin

LSD- lysosomal storage disease

GVHD- graft versus host desease

BMSCs- bone marrow mesenchymal stromal cells

ADMSCs- adipose-derived mesenchymal stromal cells

SSEA- stage specific embryonal antigen

OI- osteogenesis imperfecta

SLs- sphingolipids

SM- sphingomielin

aSMases- acid sphingomielinases

nSMases- neutral sphingomielinases

GSIs- glycosphingolipids

ER- endoplasmic reticulum

S1P- phingosine 1 phosphate

C1P- ceramide 1 phosphate

PKC- protein kinase C

KSR- kinase suppressor of Ras

TNF- tumor necrosis factor

ROS- reactive oxygen species

PP1- protein phosphatase 1

GLUT4- glucose transporter tpe 4

SK- sphingosine kinase

CFTR- cystic fibrosis tranmembrane conductance regulator

cAMP- cyclic adenosine monophosphate

IL-8- interleukin 8

NOD-SCID- non-obese diabetic-severe combined immunodeficient

GlcCer- glucosylceramide

GalCer- galactosylceramide

Gb3- globotryaosylceramide

HIV- human immunodeficiency virus

LacNac- N acetyl D-lactosamine

NKCs- natural killer cells

iNKCs- invariant NKCs

Siglec- sialic acid binding immunoglobulin like lectin

IR- insulin receptor

EGFR- epidermal growth factor receptor

VEGFR- vascular endothelial growth factor

MAPK- mitogen-activated protein kinase

HLH- helix-loop-helix

TGF- tumor growth factor

AIF-apoptosis inducing factor

CHAPTER 1 - INTRODUCTION

ADULT STEM CELLS

Stem cells are undifferentiated cells with the ability to self-renew and differentiate into cells of different organs and tissues.[1] Besides embryonic stem cells (ES), pluripotent cells derived from the inner cell mass of the embryo at E 3,5 and E 4,5 in mouse and man respectively, different stem cells populations can be extracted from many adult and neonatal tissues.[2] (fig. 1)

These adult stem cells were originally known to be multipotent as they could differentiate into the cell types of the tissue of origin.[3] (fig. 2) However, it is now accepted that they possess a certain "plasticity", that is that they demonstrate the ability to differentiate also into cells of tissues and organs different from that of origin. [4,5]

Among various adult stem cells populations, hematopoietic stem cells (HSCs) were the first isolated and characterized. HSCs reside principally in bone marrow but they can be found also in peripheral blood and umbilical cord blood (UCB) and they are known to be the precursors of all blood cells.[6]

In addition to HSCs, bone marrow is home to mesenchymal stromal cells, (MSCs) multipotent cells responsible to regenerate cells of mesenchymal origin in bone marrow.[7] MSCs can be found also in many other adult and neonatal tissues, including adipose tissue, skeletal muscle, umbilical cord and placenta and they show the ability to differentiate into cells of mesodermal and non mesodermal tissues.[5,8]

Unexpectedly, adult stem cells populations were found also in tissues known for their low capacity to regenerate. For example, neural stem cells (NSCs), multipotent cells with the ability to generate neurons and glial cells were found in the subventricular zone of the lateral ventricles and in the hippocampus of postnatal and adult mammalians brain.[9]

Many other stem cells types were then identified in other tissues, including intestinal stem cells[10] and mammary stem cells[11] Recently, very small embryonic-like stem cells (VSELs), pluripotent cells expressing ES markers, such as Oct3 and Nanog, were found in mouse bone marrow, brain, kidney, skeletal muscle and in human UCB. [12,13]

Adult stem cells reside in particular niches of the tissue of origin and they differentiate to replace senescent or damaged cells in response to specific stimuli. Moreover, they proliferate to self-renew so that the pool of tissue undifferentiated cells is maintained.[14] On this purpose, a dividing stem cell can give rise to 1) two daughters identical stem cells (symmetric division), 2) two committed progenitor cells or 3) one stem cell and one committed progenitor cell (asymmetric division).[15,16]

A complex loop of genetic determinants and signaling factors are known to be involved in maintaining the delicate balance between stem cells self-renewal and differentiation. In this direction, it was demonstrated that wnt proteins, a family of secreted molecules involved in many developmental processes, in both vertebrates and invertebrates, contribute to the regulation of stem cells self-renewal in the hematopoietic system.[17] Their mechanism of action provides binding to specific membrane receptors that leads to the stabilization

and accumulation of β-Catenin in the cytosol. Consequently, β-Catenin can migrate to the nucleus and bind specific transcription factors to regulate target genes expression.[18] Recently, the involvement of various microRNAs in stem cells gene expression regulation was studied in different pathways. For example, it was shown that miR-489 is able to maintain skeletal muscle satellite cells in a quiescence state and that, accordingly, its down-regulation results in satellite cells activation.

Moreover, miR-1 and miR-206 were up-regulated during satellite cells differentiation.[19] Again, overexpression of miR-125b in skin stem cells promoted cell proliferation with a consequent increase of stem cells pool.[20]

In addition, it was demonstrated that BMPs, a class of proteins belonging to the transforming growth factor β (TGF-β) superfamily, play important roles in regulation of stem cells properties.[21] Their function resulted to be different according to the stem cells compartment, as BMPs signal induced osteoblastic differentiation in MSCs[22] while it inhibited cells activation and proliferation in intestinal stem cells.[23] Another class of signaling molecules playing key roles in stem cells fate decision is constituted by tyrosine kinase receptors. Indeed, fibroblast growth factor (FGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors are able to regulate proliferation and differentiation in both ES and adult stem cells.[24]

For example, *in vitro* studies demonstrated that FGF and EGF increase MSCs proliferation potential[25] and EGF promotes also MSCs motility.[26]

Furthermore, it was observed that Hedgehog proteins that are involved in various aspects of embryonic development, can promote the proliferation of adult stem cells from various tissues, including HSCs[27] and NSCs.[28]

Understanding the mechanisms regulating stem cells behavior is one of the main goal of researchers who are studying possible applications of these cells in regenerative medicine and cellular therapy. To this purpose, three principal obstacles have to be overcome: first, the directed differentiation of stem cells to the desired cell type. Second, acheving high survival and functionality of cells after transplantation/infusion and third, avoiding undesired and uncontrolled proliferation of transplanted/infused cells.[29]

In this regard, together with the studies above mentioned, many other experiments were made using natural and synthetic molecules to control stem cells proliferation and differentiation. Accordingly, it was observed that calmodulin binding motif (IQ-1), a newly discovered molecule, acts on wnt signaling pathway to prevent stem cells differentiation and maintain cells in a quiescent state till required.[30] Conversely, the activation of core binding factor subunit alpha-1/runt related transcription factor 2 (CBFA/RUNX2) and osterix were seen to promote osteogenic differentiation of stem cells.[31]

Various known and new chemical compounds were identified as modulators of stem cells fate. For example, Young and colleagues demonstrated that murine and human HSCs, cultured with histone deacetylase (HDAC) inhibitors, maintain a more primitive phenotype then control cultures.[32] Interestingly, in an non-obese diabetic-severe combined immunodeficient (NOD-SCID)

repopulation assay, cells exposed to the HDAC inhibitor chlamydocin for 24 hours displayed an average of four-fold higher engrafting ability over untreated cells.[33] Moreover, from a screen of a heterocycle library, it was found a purine derivative named SR1 that increases the total number of cord blood-derived HSCs more than fifty-fold compared to controls.[34]

In MSCs, it was observed that a trisubstituited purine, named purmorphamine, exerts a potent pro-differentiating activity toward osteoblasts by upregulating CBFA/RUNX2 expression.[35]

In addition, a variety of other small molecules and chemicals with MSCs differentiation inducing activity were identified. For instance, 5-Aza-2'deoxycytidine (decitabine, trade name Dacogen) promoted the multi-lineages differentiation of mesenchymal precursor cell line C3H10T1/2 into osteoblasts, adipocytes and chondrocytes, by converting the cells into a competent spontaneous differentiation state accounting for its pleiotropic effect.[36] Furthermore, it was shown that *reversine*, a synthetic purine known for its potential of reversing adult cells into a multipotent state[37,38,39] is able to increase differentiation potential of bone marrow (B) and adipose-derived (AD) MSCs.[40]

To discover novel mechanisms involved in NSCs self-renewal, Diamandis and colleagues conducted a cell-based screen with a library of active compounds for those that inhibited neurospheres proliferation of mouse NCSs. Various compounds were found, including dihydrocapsaicin and apomorphine, agonists of the vanilloid receptor and of the dopamine receptor, respectively.[41] Other molecules were then investigated for their role in NSCs differentiation. On this line, the natural product forskolin (FSK) demonstrated the ability to

promote neurogenesis in NSCs by blocking hedgehog signaling.[42] Moreover, a 2-substituted aminothiazol named neuropathiazol induced the differentiation into βIII-tubulin positive neurons of up to 80% of cells.[33]

Recently, apart from proteins and chemicals, mechanical means were observed to be involved in stem cells differentiation. Indeed, physical properties of extracellular matrix were modulated to control osteogenic and chondrogenic differentiation of stem cells.[43] Furthermore, it was reported that manipulation of the membrane potential of cultured MSCs can influence their fate and differentiation.[44]

Although, to the present, researchers are not completely able to control and drive stem cells fate, the use of these cells to treat different pathologies in animal models or in humans showed encouraging results. In this regard, fetal and adult NSCs were used to repair damaged nervous tissue in mice presenting different brain affecting diseases.[7] In an animal model of Parkinson, NSCs transplantation led to significant cellular and functional improvement.[45] Moreover, in an animal model of Alzheimer, injection of NSCs into the basal part of forebrain led to an increase of cholinergic neurons and to an improve of cognitive ability respect to untreated controls.[46] Other studies were conducted on using NSCs in spinal cord injury. In this direction, Yan and colleagues injected NSCs into the spinal cord of rats with spinal cord injury and observed that these cells differentiated into neurons and created axons and synapses with host neurons.[47]

HSCs were mostly utilized to treat various blood and bone marrow related cancers, such as leukemia and multiple myeloma. An analysis of 586 adult T-

cell leukemia-lymphoma patients who received allogeneic HSCs transplantation revealed that HSCs therapy was effective for improving long-term survival in these patients.[48] Furthermore, HSCs transplantation was tested also in other experimental therapies, including lysosomal storage diseases (LSDs), Hurler syndrome and X-linked adrenoleukodistrophy, leading to improved patients survival rates.[7] However, these promising results were offset by several negative side effects, including graft versus host disease (GVHD) which may lead to lethal complications in patients.[49] Hence, further studies will be essential for enhancing the safety and effectiveness of HSCs transplantation.

MSCs were largely studied and analyzed for their potential in many cell therapy and medicine regenerative approaches. Their features, potentiality and use are discussed in detail in the next session.

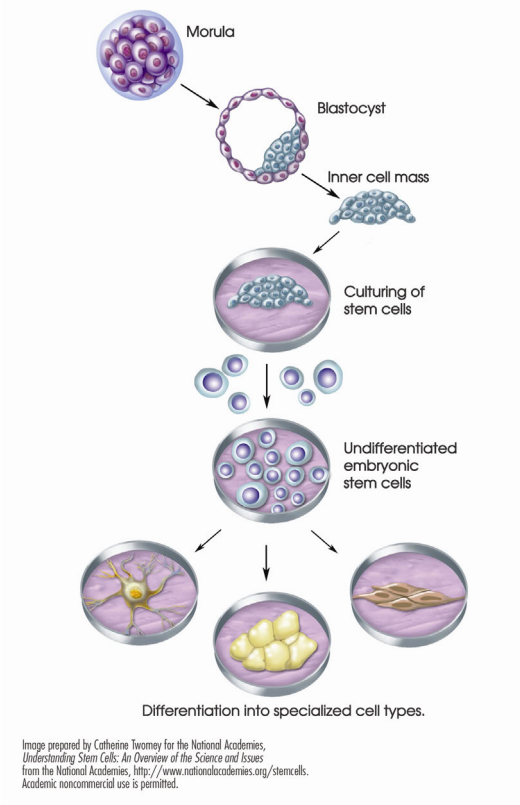

Figure 1 Embryonic stem cells isolation and differentiation

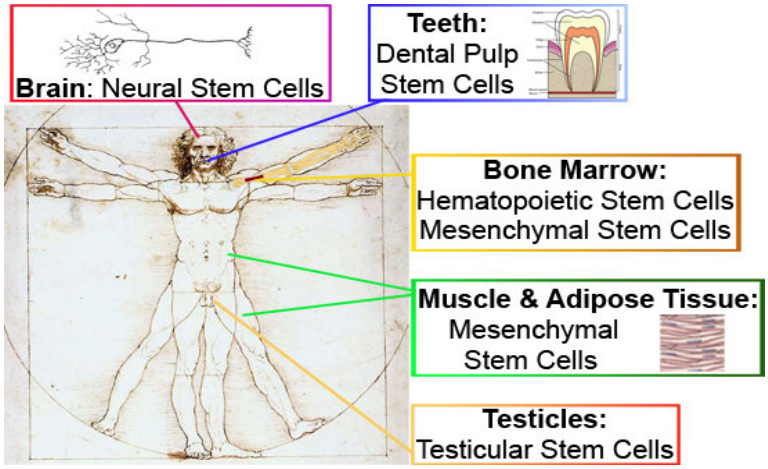

Figure 2 Examples of human adult stem cells sources

MESENCHYMAL STROMAL CELLS

MSCs were first described about forty years ago by Friedenstein and colleagues, and they were defined as *adherent cells with a fibroblast-like appearance capable of differentiating into osteocytes, chondrocytes, adipocytes, tenocytes and myocytes*.[8,50]

Today, MSCs can be isolated from almost any adult tissues, including bone marrow, adipose tissue, tendon, skeletal muscle, skin and liver.[51,52] At the same time, they can be obtained from some neonatal tissues, including: umbilical cord (UC), umbilical cord blood (UCB) and particular parts of the placenta.[53,54] MSCs derived from different sources show similar cellular and molecular features. However, many publications reported that they have some differences in their proliferation rates, surface markers expression and, most importantly, multipotency. Normally, as possibly expected, MSCs obtained from neonatal tissues show higher expansion and engraftment capacities compared to MSCs derived from adult tissues.[52] On the other hand, the success rate of cell isolation from UCB is normally lower when compared to those from adult bone marrow and adipose tissue.[55] In addition, Al-Nbaheen and colleagues reported that MSCs derived form different adult tissues showed heterogeneity in surface marker expression. For example, ADMSCs expressed notably lower levels of CD146 marker in respect to BMSCs and skin MSCs. Moreover, it was observed that BMSCs possess a higher differentiation potential toward osteoblasts than toward adipocytes while skin MSCs act in the opposite manner.[52,56]

Interestingly, it has been reported that heterogeneity in cellular morphology, differential markers expression and variable differentiation potential, occurs also within a single MSCs population as a possible reflex of the repertoire of distinct subpopulations existing *in vivo.*[57] In this direction, it was demonstrated that MSCs of a single cell-derived colony, simultaneously express mRNAs characteristic of various committed mesenchymal cell lineages including osteoblasts, chondrocytes and myoblasts.[58]

Post and colleagues provided evidence that BMSCs contain lineage-committed populations of pre-osteoblastic and pre-adipocytic cells. The authors isolated two clonal cell lines called mMSCs1 and mMSCs2 from murine bone marrow. MMSCs1 presented a round morphology and they were able to differentiate toward adipocytes, but failed the differentiation toward osteoblasts. On the contrary, mMSCs2 had a fibroblast-like morphology and they were able to differentiate toward osteoblasts, but failed to differentiate toward adipocytes.[59] This behavior suggests the presence of a lineage "imprinting" in different stromal cells compartments that influences the differentiation potential of MSCs.

Along this line, it was demonstrated that human BMSCs possess specific features according to their in situ localization; for example, the expression of CD146 marker was found in endosteally localized CD271+ MSCs but lacks in perivascular CD271+ MSCs.[60]

On the other hand, heterogeneity within a single MSCs population could be the effect of the alterations induced by extensive *ex vivo* culturing.

In this direction, it was observed that long term culture induces MSCs to change surface molecules expression profiles, to decrease the ability to differentiate toward mesodermal lineages[61,62] and to increase cell size and signs of cell aging.[63] Long term culture was further associated with continuous changes in the global gene expression profile[64] and DNA methylation,[65] accounting for the phenotypic variations occurring in these cells. Moreover, during cell isolation process, the detachment of MSCs from their original niche could induce partial cell differentiation, yielding a mixture of cells at various degree of maturation and consequently variable in morphology and phenotype.[64]

In addition to their heterogeneity, the lack of universally accepted criteria to define and characterize MSCs, had fatherly complicated the study of these cells, leading to incongruences among investigators. To address this problem, the Mesenchymal and Tissue Stem Cell Committee of the ISCT recently proposed a set of standards to define human MSCs.

First, MSCs must be plastic-adherent when maintained in standard *in vitro* culture conditions. Second, ≥95% of the MSCs population must express CD105, CD73, CD90 and must lack the expression (≤ 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, as measured by flowcytometry. Third, the cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts under *in vitro* standard differentiating conditions.[66]

Methodology employed in the isolation and enrichment of MSCs is reliant to the ability of these cells to adhere to and subsequently proliferate on tissue

culture plastic. Pre-enrichment using cocktails of antibodies or Ficoll gradient separation, that deplete contaminating cell populations, are the most widely used means for MSCs isolation.[67] In recent years, there was great interest in founding cellular markers that can strictly characterize MSCs for their direct isolation from tissues. To this purpose, some cellular surface markers were proposed, including stage-specific embryonic antigen- (SSEA-) 1 in mice and SSEA-4 or STRO-1 in humans.[68,69,70]

Quirici and colleagues used CD271 as a marker for direct isolation of human BMSCs and ADMSCs. The authors showed that the immunomagnetic sorting of MSCs labeled with antibodies against CD271 allows the selection of phenotypically and functionally homogeneous cells that are capable of expansion, self-renewal, and differentiation into multiple mesenchymal lineages.[71,72]

However, to date, there are not any available effective and reproducible methods for purification of MSCs, as no specific surface markers were found. Indeed, currently used markers are also expressed by other cells, including stromal and dermal fibroblasts.[73]

The multipotent differentiation ability of MSCs is a very important feature of these cells. Due to their mesodermal origin, MSCs were regarded as an attractive source for the generation of cells of mesodermal origin such as osteoblasts, adipocytes and chondroblasts.[74] However, recent publications reported that different MSCs population have also the potential to differentiate into several additional cell types, including skeleton[75], smooth[76] and cardiac muscle cells.[77]

Interestingly, MSCs could generate neuron-like cells *in vitro*[78] and astrocytes and neurons after implanting into the mouse brain *in vivo.*[79] Moreover, Jiang and colleagues demonstrated that BMSCs, transplanted in a non-damaged recipient, engraft and differentiate to the hematopoietic lineage, in addition to the ephitelium of liver, lung and gut. Accordingly, it was observed that BMSCs, injected in a murine blastocyst, contribute to most somatic cell types.[5]

Despite the above discussed difficulties in MSCs isolation, characterization and manipulation, their multi-lineages differentiation ability gives to these cells a great potential for clinical application in regenerative medicine and cell therapy. On this line, it was recently shown that MSCs can also secrete a variety of pro-regenerative molecules that stimulate survival and proliferation of cells and increase angiogenesis in the injured tissue.[80]

Animal models provided a useful tool for defining a number of diverse prospective applications for MSCs in tissues repair.

For example, MSCs loaded onto a porous ceramic cylinder provided significant healing potential in critical size bone defects in the canine model.[81] Furthermore, MSCs loaded onto macroporous hydroxyapatite scaffods promoted full limb functional recovery in a significantly shorter period than for traditional bone grafting.[82] Wakitani ad colleagues utilized MSCs to treat full thickness cartilage defects in a rabbit model obtaining the total repair of subchondral bone two weeks after implantation.[83]

In comparison to animal studies, there are less reports outlining the role of MSCs in promoting bone and cartilage repair in the human clinical setting. However, some positive results were obtained including two patients with full

thickness articular patellar defects and an athlete with a full thickness articular cartilage defect of the femoral condyle.[84,85]

In late years, researchers studied the role of MCSs in repairing many other different tissues, such as cerebral and cardiac tissue. In a rat model of middle cerebral occlusion, MSCs administration led to significant reduction in gross lesion volume and improved functional recovery.[86] Furthermore, in a clinical trial, autologous transplantation of MSCs in 30 patients with middle cerebral artery infarcts and neurological deficits resulted in evident improvement, without any adverse reactions after serial follow-up evaluations.[87] Other clinical and experimental evidences indicated that MSCs are a promising cell type for the treatment of cardiac dysfunction, independently from their myogenic differentiation capacity. Indeed, their beneficial effect was attributed in part to their propensity to secrete pro-survival and pro-angiogenetic factors.[88,89]

In this direction, in a randomized double-blinded study, patients receiving infusions of MSCs post myocardial infarction demonstrated significant enhancement in cardiovascular function.[90] Moreover, similar therapeutic benefits were reported in patients receiving intracorony MSCs administration compared to placebo.[91]

Allogenic transplantation of MSCs was useful also in treating genetic bone disorders, such as osteogenesis imperfecta (OI) and hypophosphatasia. *In vivo* murine models of OI demonstrated selective incorporation of MSCs in bone tissue with subsequently reduced fracture rates and increased bone strength.[92] Successful in utero-transplantation of MSCs was reported in a human fetus

with severe OI who after birth demonstrated regular growth and psycomotor development.[93]

In addition, thanks to their remarkable immunosuppressive properties, MSCs were used to treat immune-associated conditions such as GVHD Indeed, MSCs were known to suppress T-lymphocytes and NKCs functions and to induce regulatory T lymphocytes activation.[94] Furthermore, these cells could secrete soluble factors that inhibit B lymphocytes differentiation and impair their chemotaxis.[95]

Animal models of GVHD suggested that BMSCs and ADMSCs have the same immunosuppressive effect and lead to significant symptomatic improvement.[96,97] Moreover, encouraging results were obtained also in human; in a phase II clinical trial on patients with steroid-resistant, acute GVHD, MSCs treatment led to lower transplantation related mortality and higher two-year survival post MSCs transplantation.[98]

The promising results obtained with clinical applications of MSCs induced many researchers to focus their attention on the study of these cells and their possible application. In this context, this Ph.D. project was dedicated to the research of new possible surface markers for the identification, characterization and possibly isolation of human BMSCs. Moreover, new candidate molecules involved in the differentiation of MSCs were investigated.

In this direction, we focused our attention on sphingolipids, a family of lipids found in the outer leaflet of cell plasma membranes and involved in many cell signaling pathways. The physiological and pathological role of sphingolipids in

the cell, which is the basis of the hypothesis of their involvement in stem cell differentiation, are discussed in the next section of this thesis.

SPHINGOLIPIDS

Sphingolipids (SLs) are a class of lipids composed by a long-chain sphingoid base as backbone. Sphinganine and sphingosine are the most common sphingoid bases found in SLs even if others were described, including phytosphingosine and methylsphingosine.[99]

Ceramide, the simplest SL, consists of a sphingoid base to which a fatty acid is attached at C-2 via N-acylation. In nature, different ceramides exist depending on the N-fatty acyl chain that can contain from two to twenty-eight carbon atoms.[100] In the cell, ceramide synthesis occurs in the cytosolic leaflet of the endoplamic reticulum (ER) and begins with the condensation of serine and palmitoyl CoA to form 3-ketosphinganine, which is subsequently reduced to produce sphinganine. Dihydroceramide, formed by sphinganine acylation is then converted into ceramide by the insert of a *trans* 4-5 double bond.[101]

In addition to *de novo* synthesis above mentioned, ceramide can be also generated from rapid hydrolysis of sphingomyelin (SM) by acid sphyngomyelinases (aSMases) in the lysosome or neutral sphyngomyelinases (nSMases) at the plasma membrane.[102] Moreover, besides these pathways, other observations provided evidence for ceramide generation from the processing glycophigolipids (GSLs) at the plasma membrane.[103]

Ceramide is the key hub in the SL biosynthetic pathway and it may be converted into a variety of metabolites. Deacylation by ceramidases enzymes acting in different cell compartments, including lysosomes,[104] plasma membrane[105] and endoplasmic reticulum (ER)/Golgi complex,[106] yields sphingosine, which may be phosphorylated by sphingosine kinase to sphingosine-1-phosphate (S1P).

In addition, ceramide may be converted to ceramide-1-phosphate by ceramide kinase (C1P) or transported to the Golgi complex to be coverted to SM by the tranfer of phosphorylcoline via SM synthase.[102] The importance of the equilibrium between SLs biosynthesis and degradation is underlined by different diseases associated with defects of the enzymes involved in these pathways.

In this regard, mutations in SMPD1 gene encoding aSMase cause different forms of Niemann-Pick disease, including type A characterized by severe neurovisceral phenotype and type B exhibiting little neural degeneration with frequent liver and spleen pathologies.[107] Moreover, deficiencies in acid ceramidases lead to Farber disease, a disorder characterized by the accumulation of ceramide in cells with consequent impairment of tissues functionality.[108]

SLs are fundamental structural components of cell membranes as well as mediators of cell signaling of different physiological and stress stimuli.[102]

The main pathways in which SLs are involved are:

CELL DEATH PATHWAY. Ceramide is involved in cell growth arrest and apoptosis, through different mechanisms of action: first, it acts as a second messenger binding several intracellular targets, including the protein kinase C (PKC),[109] kinase suppressor of Ras (KSR)[110] and Raf-1.[111] In this direction, it was demonstrated that ceramide mediates tumor necrosis factor- (TNF-) induced cell death by activating directly endosomal protease cathepsin D. Direct binding of ceramide to cathepsin D resulted in autocatalytic proteolysis of the 52 kDa pre-pro cathepsin D to form the enzymatically active 48/32 kDa isoforms.[112]

Second, ceramide could act directly on mitochondria interacting with components of the mitochondrial electron transport chain, accounting for the stimulation of ROS production and mitochondrial depolarization observed during apoptosis.[100] Indeed, in cell-free assays using purified rat liver mitochondria, it was shown that the addition of ceramide directly induces a burst of ROS generation and blockade of the mitochondrial electron transfer.[113,114]

Third, ceramide, generated within membrane lipid rafts through SMases action, could self-associate and induce raft coalescence into large transmembrane signaling platforms that are involved in death signaling for diverse cellular stresses, such as physical stresses, bacterial and viral infections, chemotherapy and inflammatory mediators.[115,116]

PRO-SURVIVAL PATHWAY. In contrast to ceramide, S1P and C1P function as pro-survival molecules. It was shown that S1P act as second messenger to

mediate calcium homeostasis, cell growth and suppression of apoptosis.[117] Interestingly, it was postulated that S1P could also have trophic effects on skeletal muscle. In this regard, it was demonstrated that exogenous application of S1P counteracts the reduction of rat soleus muscle mass caused by denervation, whereas neutralization of the extracellular lipid with a specific anti-S1P antibody accelerates the denervation-induced atrophy.[118] C1P was observed to share similar functions of S1P, exerting its anti-apoptotic function through the inhibition of protein phosphatase 1 (PP1).[119]

PRO-ANGIOGENETIC PATHWAY. It was observed that S1P stimulates angiogenesis *in vivo* via S1P1 and S1P2 receptors in the Matrigel implant assay.[120] Moreover, it was demonstrated that, in vascular endothelial cells, S1P and its receptor S1P1 are essentially required for the recruitment process of pericytes and smooth muscle cells to the nascent capillaries and thus vascular maturation.[121,122] Accordingly, it was recently shown that intramuscular administration of S1P, in mouse hindlimb after ischemia, is useful to neo-vessels formation and consequent acceleration of blood flow recovery.[123]

The studies of physiological role of SLs were crucial to understand the contribute of SLs in various disease processes. In this regard, it was demonstrated that ceramide can inhibit insulin-stimulated glucose uptake, glucose transporter type 4 (GLUT4) translocation and glycogen synthesis, contributing to the development of insulin resistance resulting from lipid over-supply.[124] The inhibitory effect of ceramide on insulin signaling mainly resulted

from its ability to block the activation of akt/protein kinase B, a central mediator of insulin action.[125] Confirming this thesis, it was observed that in myotubes derived from C2C12 cell line, palmitate increases ceramide content with subsequent inhibition of downstream insulin-stimulated Akt phosphorylation.[126] Recently, it was shown that SK1/S1P axis is also crucially implicated in the regulation of glucose metabolism in skeletal muscle. Indeed, pharmacological or siRNA-mediated inhibition of SK1 resulted in an appreciable decrease in basal and insulin-stimulated glucose uptake in C2C12 myoblasts. Moreover, overexpression of SK1, which caused an increase of intracellular S1P levels, enhanced basal and insulin-stimulated glucose uptake.[127]

In addition, SLs were investigated for their involvement in in the pathogenesis of cystic fibrosis, an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR).[128] Indeed, ceramide produced from SM at the plasma membrane was able to inhibit cyclic adenosine monophosphate (cAMP)-mediated anion transport by CFTR.[129] Another study demonstrated that fenretinide, which upregulates *de novo* ceramide synthesis, reduces interleukin-8 (IL-8)-mediated inflammation in CFTR-deficient lung epithelial cells.[130]

As ceramide is known to be involved in cell death signaling, it was postulated that it could have anti-carcinogenic activity. In this direction, it was shown that direct administration of ceramide induces apoptosis in cancer cells and cancer cell lines.[131,132] *De novo* ceramide synthesis was altered in human head and neck carcinomas and was implicated in caspase-dependent cancer cell death

pathway.[133] Furthermore, the effectiveness of some chemotherapeutic agents appeared to be related to their ability to activate ceramide-mediated apoptosis.[134]

GLICOSPHINGOLIPIDS

The attachment of a sugar head group to ceramide gives origin to the most structurally complex class of SLs: GSLs. After being synthetized in the ER, ceramide can be delivered by vesicular transport to the Golgi apparatus where GSLs synthesis occurs. Here, the transfer of a glucose or a galactose residue by specialized glycosyltransferases to ceramide lead to the formation of glucosylceramide (GlcCer) or galactosylceramide (GalCer) respectively, the precursors of all known GSLs. GalCer can be subsequently modified into sulfatides while GlcCer can be converted into more complex GSLs through sequential transfer of sugars by different glycosyltransferases.[135] Many different carbohydrates structures were described in GSLs with the main sugars being glucose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid. Based on their core carbohydrate structures, GlcCer-derived GSLs are classified in different series, the main common being ganglio- (Galß1-3GalNAcß1-4Galß1-4Glcß1-1'Cer), lacto- (Galß1-3GlcNAcß1-3Galß1-4Glcß1-1'Cer) and globo-(GalNAcß1-3Gala1-4Galß1-4Glcß1-1'Cer) series. Moreover, they can be subdivided into neutral and acidic GSLs. Acidic GSls containing

one or more sialic acid residues are referred to as gangliosides as they are particularly abundant in nervous tissue.[136]

The catabolism of GSLs proceeds by stepwise, sequential removal of sugars by lysosomal exohydrolases to the final common products, sphingosine and fatty acids. In this process, non-enzymatic proteins such as saponins are essential either by presenting lipid substrates to their cognate enzymes, or by interacting with specific enzymes.[137]

Various severe diseases caused by deficit in GSLs metabolism were described. On this line, disrupted gangliosides synthesis in mice with either GM3 synthase,[138] GM2/GD2 synthase[139] or GD3 synthase[140] knocked out, caused differential neurological impairments. Again, in mice with GlcCer synthase knocked out, it was shown early embryonic lethality.[141] In humans, a loss-of-function mutation in GM3 synthase gene was identified in a cohort characterized by autosomal recessive infantile-onset epilepsy syndrome.[142] Moreover, a patient with GM2/GD2 synthase deficiency died at three month after presenting abnormal motor function and seizures.[143] Conversely, defects in GSLs hydrolases result in excessive accumulation of specific GSLs in lysosomes leading to various LSDs. For example, Fabry disease, due to the deficiency of alpha-galactosidase, the enzyme that cleaves principally globotriaosylceramide (Gb3), is characterized by Gb3 accumulation in many tissues and organs leading mainly to cardiac and cerebrovascular complications.[144] Tay-Sach disease is another known LSD caused by defect of β-hexosaminidase A with consequent GM2 accumulation leading to neurological complications.[145]

GSIs are localized in cellular membranes and principally at the plasma membrane.

Here, the long hydrocarbon chains of ceramide drive them to partition laterally into lipid rafts, cholesterol-rich membrane microdomains[146] known for their involvement in cell signaling transduction. GSLs bind many different interactors and are involved in many different physiological and pathological signaling pathways acting as:

VIRUSES AND TOXINS RECEPTORS. It has long been known that GSIs can act as receptors for viruses and toxins promoting their internalization into the cell. In this regard, it was demonstrated that acid sialic residues on gangliosides participate in influenza virus internalization,[147] whereas some cell surface GSLs interact with different components of the human immunodeficiency virus (HIV) fusion machinery.[148] Moreover, it was observed that ganglioside GM1 and Gb3 function as receptors for cholera toxin and shiga toxin respectively, promoting their internalization by the cell.[149,150]

GSLs were then investigated also for their involvement in post-infectious disease, such as Guillain-Barrè or Miller-Fisher syndromes. Indeed, IgG antibodies directed against pathogen antigens such as lipopolisaccaride of C. jejuni cross-react with host gangliosides causing autoimmune pathologies.[151]

CELL-CELL INTERACTION MEDIATORS. GSIs are involved in glycan-driven cell-cell recognition systems through the interaction with other GSIs or with lectins.

It is known that lectins E-selectin and P-selectin expressed on the endothelium bind to sialic acid- and fucose-containing glycans on neutrofils, event that promotes neutrofil adhesion to endothelium and squeeze into the surrounding tissue.[152] Interestingly, it was shown that while P-selectin binds to glycoproteins, E-selectin receptors are fucosylated gangliosides with very long chain poly N-acetyl-D-lactosamine (LacNac) structures.[132] Furthermore, it was demonstrated that natural killer cells (NKCs) express a specific lectin named Siglec- (sialic-acid binding immunoglobulin-like lectin-) 7, which binds preferentially b-series gangliosides expressed on other cells with consequent modulation of NKCs activity.[153] Indeed, cells overexpressing the b-serie ganglioside GD3 suppressed NKCs-mediated cytotoxicity in a siglec-7 dependent manner.[154] Another member of siglec family, siglec 4, is known to be expressed in nerve tissue by cells that form myelin[155] (where it binds to gangliosides to stabilize axon-myelin interaction and regulate axon outgrowth after injury. As confirmation, B4galnt1- (GM2/GD2 synthase) null mice displayed axon degeneration and dysmyelination similar to that found in sigle-4-null mice.[156] In addition, St3gal5/B4galnt1 (GM3 and GM2/GD2 synthases) double-null mice showed severe disruptions in axon-myelin interactions.[157]

MEMBRANE RECEPTORS MODULATORS. Modulation of GSLs expression can have profound effects on the activity of various tyrosine kinases receptors, including insulin receptor (IR), epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR).

IR regulation by gangliosides was demonstrated in various systems: first, addition of gangliosides to partially purified IRs reduced insulin-stimulated tyrosine phosphorylation.[158] Second, in a cellular model of insuline resistance, the attenuation of IR signaling, induced by TNF-alpha, was accompanied by elevated GM3 biosynthesis. Moreover, pharmacological block of gangliosides biosynthesis could reverse the effect of TNF-alpha.[132] Third, in an *in vivo* model, St3gal5- (GM3 synthase) null mice, which lack GM3 and downstream gangliosides, had enhanced insulin sensitivity with increased IR phosphorylation in skeletal muscle.[159] GM3 action on IR inhibition, appears to be due to a direct binding ganglioside-receptor as a site-directed mutagenesis study identified a cationic residue (Lys-944) required for IR-GM3 interaction.[160]

In addition to its effect on insulin signaling, ganglioside GM3 has long been known to inhibit EGFR tyrosine kinase without interfering with EGF binding.[161]

A recent study by Coskun and colleagues demonstrated that GM3 limits receptor dimerization and autophosphorylation. This GM3-mediated inhibition can be overcome by the EGF ligand and relies on the direct interaction between GM3 sialic acid with an extracellular lysine localized proximally to the EGFR transmembrane domain[162]

Gangliosides participate also in VEGFR signaling. Indeed, addition of ganglioside GD1a enhanced VEGF-induced VEGFR phosphorylation with consequent proliferation of vascular endothelial cells *in vitro.*[163] Moreover, GD1a increased the expression of VEGFR and reduced the concentration of VEGF required to induce receptor activation. On the contrary, ganglioside GM3

was able to inhibit VEGFR phosphorylation and to reduce VEGF-induced proliferation *in vitro* and angiogenesis *in vivo.*[164]

EXTRACELLULAR AND INTRACELLULAR CELL SIGNALING MEDIATORS. α-GalCer, a GSL derived from marine sponge, is able to bind and activate a special subset of immune T cells, invariant NKCs (iNKCs). Accordingly, it was observed that following α-GalCer binding, iNKCs rapidly secreted IL-4 and interferon-γ with consequent transactivation of various cells of the innate and adaptive immune system.[165]

Gangliosides were also proposed as intracellular mediators involved in different cellular pathways. Ganglioside GD3 was known to act as pro-apoptotic factor in different cell types. Indeed, in human hemopoietic cell lines, GD3 rapidly accumulated upon CD95 triggering or ceramide stimulation with consequent cell apoptosis. In addition, CD95- and ceramide-induced apoptosis could be blocked if endogenous GD3 accumulation was prevented by suppressing GD3 synthase expression.[166] It was demonstrated that the pro-apoptotic function of GD3 is explicated at different levels: it can directly target mitochondria inducing the loss of mitochondrial potential, release of ROS followed by cytocrome C release and caspases activation.[167] Moreover, GD3 can block the activation of the nuclear receptor NF-kB and consequently the transcription of NF-kB-dependent pro-survival genes by preventing NF-kB translocation from the cytosol to the cell nucleus.[168]

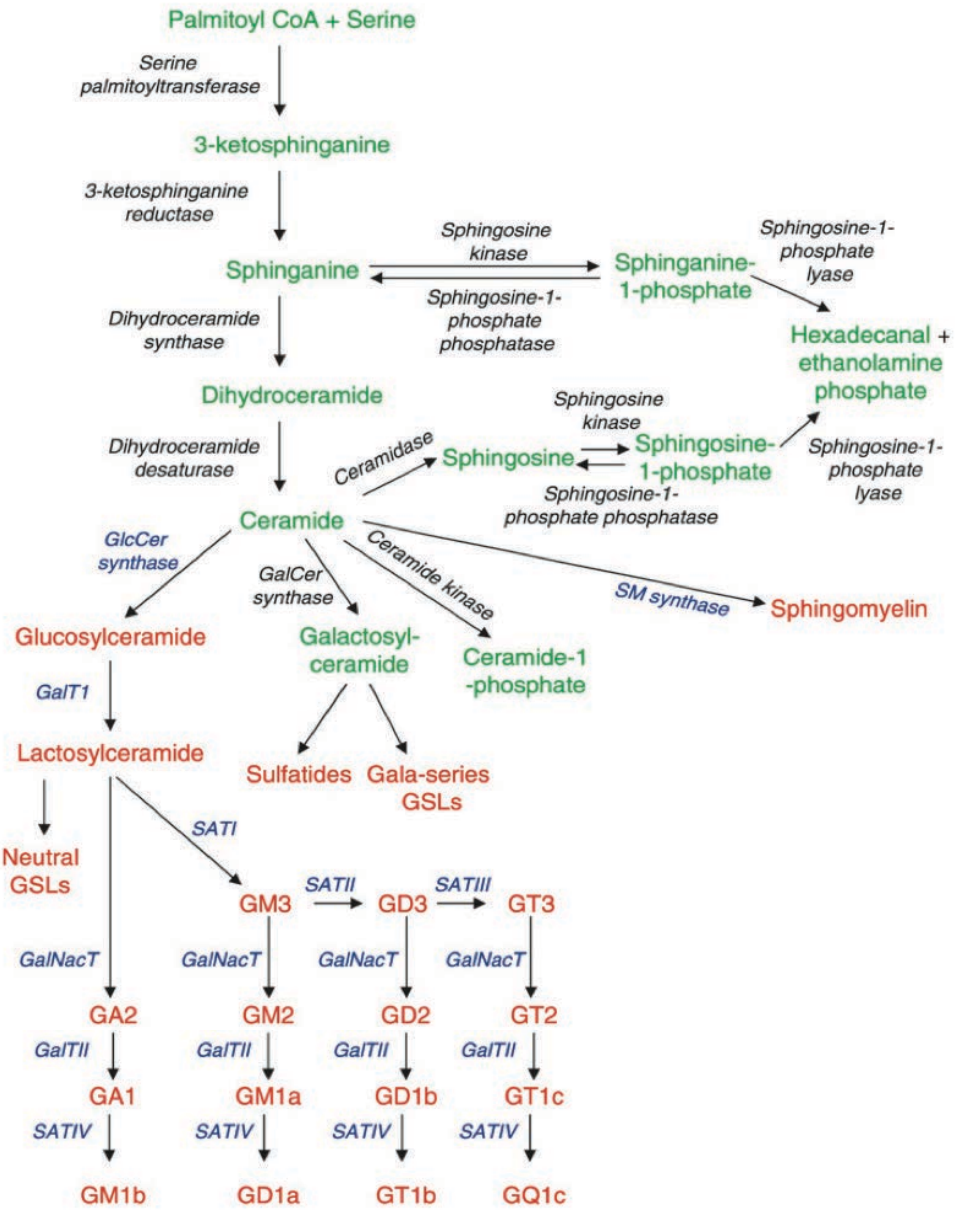

Figure 3 The metabolism of SLs and GSLs in the ER and Golgi apparatus.

GSLs AND ADULT STEM CELLS

Recently, researchers developed great interest in the study of GSLs expression and role in stem cells. Because of their principal localization in the outer leaflet of plasma membranes, GSLs were seen as possible marker molecules for different stem cells characterization and isolation. Moreover, it was shown that GSls are involved in many different stem cells signaling pathways, including proliferation, differentiation and apoptosis. The main studies relating GSLs in NSCs, HSCs and MSCs are discussed below.

NEURAL STEM CELLS

It was reported that NSCs express principally b-series gangliosides such as GD2, detected only by immunological techniques[169] and GD3, biochemically detected by thin-layer chromatography. Along this line, ganglioside GD3 was then proposed by Nakatani and colleagues as unique and powerful cell-surface marker to identify and isolate NSCs from brain. In this work, they demonstrated that GD3 is expressed in more than 80% of NCSs prepared from postnatal and adult mouse brain tissues and its expression decreases drastically with cell differentiation. Moreover, $GD3^+$ cells isolated from postnatal and adult mouse subventricular zones, efficiently generated neurospheres compared to $GD3^-$ cells and possessed multipotency to differentiate into neurons, astrocytes and oligodendrocytes.[9]

SSEA-1 and SSEA-4 antigens were also investigated as possible NSCs specific surface markers. In this direction, NSCs prepared from mouse brains at postnatal day 1 and from human brains at gestational week 17 expressed SSEA-1 by flow cytometry analysis.[170] Furthermore, SSEA-1$^+$ cells isolated from the subventricular zone had characteristics of NSCs.[171]

In human forebrains, SSEA-4 was found expressed in CD133$^+$ NSCs and CD133$^+$/SSEA-4$^+$ cells isolated by FACS were able to form neurospheres and to differentiate to neurons and astrocytes.[172]

GSLs resulted useful for isolating not only NSCs but also glial precursor cells, progenitors cells that could differentiate to type-2 astrocytes and oligodendrocytes. Indeed, GT3 and O-acetyl GT3 were recognized as glial precursor cells markers and they were used to enrich these cells from a heterogeneous population of rat NSCs by magnetic cell sorting.[173] GSLs were then studied for their role in regulating NSCs behavior and differentiation. In this regard, gangliosides pattern of neuroepitelial cells was modulated during neuronal differentiation. Indeed, a-series gangliosides such as GM1 and GD1a, not expressed in neuroepithelial cells, became detectable after nueronal differentiation.[174] Moreover, depletion of GSLs by a GSLs synthesis inhibitor, repressed the activation of Ras-mitogen-activated protein kinase (MAPK) pathway and retarded mouse neuroepithelial cells proliferation.[175]

HEMATOPOIETIC STEM CELLS

To date, few information on HSCs GSLs are available. One of the first works on GSLs pattern of HSCs were conducted in rat lymphocyte progenitor cells

prepared from bone marrow by Ficoll gradient centrifugation. Here, gangliosides were biochemically quantified founding tetrasialogangliosides as major components and disialogangliosides as minor components.[176] Furthermore, it was found that cholera toxin, a molecular probe recognizing GM1, is reactive to mouse HSCs suggesting the presence of GM1 in these cells.[177] However, as it is known that cholera toxin also binds to a lesser extent other gangliosides such as asialo-GM1, fucosyl-GM1 and GD1a,[178] the presence of GM1 should have been confirmed by biochemical analysis.

MESENCHYMAL STROMAL CELLS

GSLs expression and role in MSCs derived from different tissues was investigated by various researchers. In this regard, it was reported by Gang and colleagues that SSEA-4 antigen can be a good marker for mouse and human BMSCs. Along this line, SSEA-4$^+$ cells isolated by FACS showed high proliferative ability and could differentiate into osteoblasts and adipocytes.[69] Subsequently, SSEA-4 expression was found also in human UCB-derived MSCs by mass spectrometry and immunological techniques. However, this marker can not be considered optimal for these cells as its expression resulted to be altered by cell culture conditions.[179]

Martinez and colleagues proposed ganglioside GD2 as a novel surface marker for human BMSCs. Using immunological techniques, they observed that MSCs are the only cells within normal marrow presenting GD2 and its expression is stably mantained after *in vitro* cells expansion. Furthermore, GD2$^+$ cells isolated from bone marrow by immunomagnetic selection presented

differentiation capacity to osteoblasts, adipocytes and chondroblasts.[180] On the same line, it was shown that GD2 is expressed in UCB-derived MSCs and it identifies a subpopulation of cells with high clonogenicity and proliferation capacity as well as strong multi-differentiation potential. $GD2^+$ sorted cells showed increased expression of typical ES markers, such as SSEA-4, Oct-4, Sox-2 and Nanog.[181] Moreover, inhibition of GD2 synthesis in UCB-derived MSCs suppressed neuronal differentiation and down-regulate helix-loop-helix (HLH) transcription factors which are involved in early neuronal differentiation.[182]

Besides their possible role of specific markers for MSCs isolation, GSLs were investigated for their possible involvement in MSCs differentiation. Different works demonstrated that GSLs pattern of MSCs significantly change when cells are induced to differentiate. In this direction, ganglioside GT1b was found in 9-old differentiated neuronal cells derived from periodontal MSCs but not in starting stem cells. In addition, it was observed that in dental pulp-derived MSCs, knockdown of GlcCer synthase by small hairpins RNAs, leading to the lack of all GSLs derived from GlcCer, causes the inhibition of neuronal differentiation.[183]

Lee and colleagues showed that human ADMSCs express gangliosides GM3, GM2 and GD1a and they registered an increase of GD1a after 5 days differentiation toward osteoblasts.[184] Moreover, in MSCs from dental pulp, it was observed an increase of ganglioside GD1a together with a decrease of ganglioside GM3 during differentiation toward osteoblasts. In addition, an increment in the activation of alkaline phosphatase, a well known marker of

osteogenic differentiation, was reported in these cells in response to treatment with GD1a together with EGF.[185] Yang and colleagues then demonstrated that inhibition of GD1a synthesis, by ST3Gall II (GD1a synthase) silencing, suppresses differentiation of human MSCs toward osteoblasts.[186]

Recently, it was speculated that S1P participates in BMSCs differentiation toward hepatic myofibroblasts through tumor growth factor (TGF) β-induced sphingosine kinase (SK) activation and S1P receptors recruitment. In favor of this hypothesis, pharmacological or siRNA inhibition of SK1 abrogated the pro-differentiating effect of TGF-β1.[187] Moreover, using S1P specific antagonists, it was demonstrated that the pro-differentiating effect of TGF-β1 is mediated by S1P receptors.[188] S1P and SM metabolism were also implicated in the activation of satellite cells, stem cells resident in skeletal muscle known to carry out muscle regeneration processes. Nagata and colleagues observed that, in quiescent satellite cells, levels of plasma membrane SM considerably decrease with consequent increase of intracellular S1P, which mediates the entry of satellite cells into the cell cycle.[189,190] Moreover it was reported that, along with a pro-myogenic effect, S1P also exerts anti-migratory action in C2C12 myoblasts needed for the establishment of cell-cell contact and fusion to form myotubes.[191]

SLs are involved also in other MSCs signaling pathway, including apoptosis. In this regard, it was observed that ceramide causes a loss of cell viability in a concentration- and time-dependent manner in ADMSCs. Furthermore, ceramide promotes ROS generation, cytocrome c release from mitochondria, caspases activation and apoptosis-inducing factor (AIF) nuclear translocation,

suggesting that these SL induces apoptosis through both caspase-dependent and caspase-independent mechanisms.[192]

AIMS

MSCs are the most studied adult stem cells, as they can be isolated from almost any tissue, they show a good self-renewal capacity in vitro and they also possess good "*plasticity*".[5] Oddly, MSCs are identified and defined by a combination of markers that are not distinctive, as they are shared by other cells including fibroblasts. Therefore, pure populations of MSCs cannot be isolated, as they are always contaminated by other adult cells that often do not possess stem cell plasticity. Thus, it would be very desirable to discover novel cell surface markers that would allow to discriminate MSCs from other cells. Moreover, new ways to control and promote cell differentiation are needed. Thus, it becomes crucial to search for new key molecules that can be modulated to increase MSCs differentiation toward the desired tissue or inhibit the differentiation when cells have to remain in an undifferentiated state.

In this direction, we focused our attention on SLs, a family of lipids found in the outer leaflet of the plasma membrane and involved in many cell signaling pathways. Therefore, main aims of this work were:

1. to investigate the possible use of SLs as new surface markers for the identification, characterization and possibly isolation of human bone marrow MSCs;
2. to investigate the involvement of SLs in the preservation of the undifferentiated state of MSCs during in vitro culturing

3. to assess the possible role of SLs in the differentiation processes of MSCs upon opportune stimuli.

CHAPTER 3 - MATERIALS AND METHODS

3.1 CELL CULTURE

Human BMSCs were grown in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (Sigma aldrich) with 10% v/v fetal bovine serum (FBS; HyClone, Thermo Scientific), 4 mM L-glutamine, 1% antibiotic-antimycotic (Euroclone) in a humidified atmosphere containing 5% CO2 at 37°C.

3.2 CELL DIFFERENTIATION TOWARD OSTEOBLASTS

Confluent BMSCs were diffentiated toward osteoblasts using DMEM low glucose 10% FBS with 0,1 uM Dexamethasone, 50 µg/ml L-Ascorbic acid 2-phosphate and 10 mM β-Glycerophosphate (Sigma Aldrich) for 17 days. At the end of differentiation process, Alizarin Red solution (Millipore) was used to detect calcium deposition by derived osteoblasts according to the following protocol:

differentiated cells were fixed using iced cold 70% ethanol for 1 hour at room temperature (RT). After two washing steps with water, cells were incubated with 1 ml of Alizarin Red solution for 30 minutes at RT. At the end of the incubation time, Alizarin Red solution was removed and cells were washed three times with water to remove the excess color. The stain associated to calcium deposition was visualized using a Axiovert 40 microscope (Carl Zeiss Microscopy) equipped with a Moticam 2300 camera (Motic)

3.3 CELL DIFFERENTIATION TOWARD ADIPOCYTES

Confluent BMSCs were differentiated toward adipocytes using Mesenchymal Stem Cells Adipogenesis Kit (Millipore) according to the manufacturer's instructions. Briefly, cells were differentiated using 1 uM dexamethasone, 500 uM 3-isobutyl-1-methylxanthine (IBMX), 10 ug/ml insuline and 100 uM indomethacine for 21 days.

At the end of the differentiation process, Oil Red O solution (Millipore) was utilized to stain lipid droplets of derived adipocytes according to the following protocol:

differentiated cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at RT. After two washing steps with water, cells were incubated with 1 ml of Oil Red O solution for 50 minutes at RT. At the end of the incubation time, Oil Red O solution was removed and cells were washed three times with water to remove the excess color. The stain of lipid droplets was visualized using a Axiovert 40 microscope equipped with a Moticam 2300 camera.

3.4 DIFFERENTIATION TOWARD SMOOTH MUSCLE CELLS

BMSCs were differentiated toward smooth muscle cells using DMEM low glucose 1% FBS with 0,2 ul/ml TGFβ1 (Millipore) for 7 days. Immunofluorescence staining was utilized to detect smooth muscle α-actin in differentiated cells according to the following protocol:

cells were fixed using 4% paraformaldeyde (Sigma Aldrich) in PBS for 10 minutes at RT. After two washing steps with PBS, aspecific binding sites were

blocked with a blocking solution (1X PSB, 1% BSA) for 1 hour at RT. Cells were incubated with the FITC-conjugated anti-human α-actin (Sigma) at the concentration of 1:200 in blocking solution for 1 hour and 15 minutes at RT. Cells nucleus were stained with Hoechst 33342 (Sigma Aldrich) at the concentration of 1:500 in blocking solution containing 0,1% Triton X100 (Sigma Aldrich) for 30 minutes at RT.

The images were acquired with a Olimpus IX51 fluorescence microscope (Olimpus).

3.5 DIFFERENTIATION TOWARD CHONDROCYTES

For chondrogenic differentiation the pellet culture method was used. BMSCs were mantained in 3-D culture in AdvanceSTEM Chondrogenic Differentiation Medium (HyClone, Thermo Scientific) for 28 days. At the end of the differentiation process, mucopolysaccharides produced by derived chondroblasts were detected by Alcian blue staining (Sigma Aldrich) according to the following protocol:

differentiated cells were layered on glass slides and they were fixed using 4% paraformaldehyde in PBS for 30 minutes at RT. After two washing steps with water, cells were incubated with Alcian blue solution for 1 hour at RT. At the end of the incubation time, Alcian Blue solution was removed and cells were washed once with 0,1M HCl and twice with water. The stain of mucopolysaccharides was visualized using a Axiovert 40 microscope equipped with a Moticam 2300 camera.

3.6 FLOW CYTOMETRY ANALYSIS

BMSCs were trypsinazed and collected in PBS at a concentration of 2 x 10^6 cells/mL. Aspecific binding sites were blocked with a blocking solution (50% 1X PSB, 50% FBS) for 30 minutes at RT. Cells were stained with antibodies against human:

PE-conjugated CD105, PE-conjugated CD90, FITC-conjugated CD73,

FITC-conjugated CD11b, PE-conjugated CD45, PerCP-eFluor 710- conjugated CD34, FITC-conjugated CD3, FITC-conjugated CD19, FITC-conjugated HLA-DR (eBioscience) for 10 minutes at 4° C. The respective isotype antibodies were used as controls.

For indirect staining, cells were incubated with primary antibodies against human gangliosides GD2 (clone 14.G2a; BD Pharmigen) or GD1a (clone GD1a-1; Millipore) for 10 minutes at 4° C and then with secondary antibodies PE-conjugated anti-mouse IgG (eBioscience) or Alexa Fluor 488-conjugated anti-mouse IgG (Jackson Immuno Research) for 10 minutes at 4° C.

Samples were acquired with Navios flow cytometer (Beckman Coulter) and analysed with Kaluza software (Beckman Coulter)

3.7 METABOLIC LABELING OF CELL SLs

[3-^{3}H]Sphingosine (Perkin Elmer) dissolved in ethanol was transferred into a glass sterile tube and dried under a nitrogen stream; the residue was then dissolved in an appropriate volume of DMEM low glucose with 10% FBS to obtain a final concentration of 2,4 nM.

The medium was added to cells and after 2 hours of incubation (pulse), it was replaced with DMEM low glucose with 10% FBS not containing radioactive sphingosine for 48 hours (chase). At the end of the chase, cells were washed and harvested in ice-cold PBS by scraping. The cell suspension was frozen and lyophilized.

3.8 EXTRACTION AND ANALYSIS OF RADIOLABELED SLs

Total lipids from lyophilized cells were extracted twice with 20:10:1 (v/v) chloroform/methanol /water and dried under a nitrogen stream.

Lipid extract were dissolved in 2:1 (v/v) chloroform/methanol and subjected to a two-phase partitioning 20% (v/v) water.

Radioactive lipids of the aqueous and organic phases obtained after partitioning were analysed by high performance thin layer chromatography (HPTLC). The solvent system used were 110:40:6 (v/v) chloroform/methanol/water and 60:40:9 (v/v) chloroform/methanol/ $CaCl_2$ 0,2% for the organic and aqueous phases respectively.

Radioactive lipids were visualized with a Beta-Imager 2000 (Biospace) and identified by comparison with radiolabeled standards. The radioactivity associated with individual lipids was determined with the specific β–Vision software (Biospace)

3.9 ANALYSIS OF ENDOGENOUS SLs

Total lipids from lyophilized cells were extracted twice with 20:10:1 (v/v) chloroform/methanol /water and dried under a nitrogen stream.

Lipid extract was dissolved in 2:1 (v/v) chloroform/methanol and subjected to a two-phase partitioning 20% (v/v) water to separate the organic phase from the aqueous phase.

Lipids of the organic phase were submitted to an alkaline treatment to remove glycerophospholipids. Breafly, the dried organic phase was resuspended in 100 ul of $CHCl_3$ and 100 ul of 0,6 N NaOH in methanol and allowed to stand at 37°C for 1 hour. The reaction was blocked by adding 120 ul of 0,5 M HCl in methanol. The sample was then submitted to another phase separation and the new organic phase was used for HPTLC analysis.

Markers and gangliosides of the aqueous phase (4 mg of protein/lane) were loaded on HPTLC plate , developed in 60:40:9 (v/v) chloroform/methanol/ 0,2% $CaCl_2$ and visualised using Earlich's reagent. Markers and lipids of the organic phase (2 mg of protein/lane) were developed in 110:40:6 (v/v) chloroform/methanol/water and visualized using Anisaldeide solution.

3.10 CELL SEPARATION BY IMMUNOBEADS

BMSCs expressing GD2 or GD1a were enriched with immunobeads technique (Miltenyi Biotech). Cells were trypsinazed and collected in washing buffer (1X PBS, 0,5% BSA, 2 mM EDTA). 2,5 x 10^6 cells were incubated with 4 ug of primary antibody against GD2 or 4 ul of primary antibody against GD1a for 15 minutes at 4°C. After two washing steps, cells were incubated with 30 ul of anti-mouse IgG microbeads (Miltenyi Biotech) for 15 minutes at 4°C. Cells were then washed twice and resuspended in 500 ul of washing buffer. The columns used for cell separation were placed in a octoMACS Separator (Miltenyi

Biotech) and prepared by rinsing 500 ul of washing buffer. The cell suspensions were added to the columns. The unlabeled cells passed through the columns and were collected while magnetically labeled cells remained attached to the columns. These cells were then eluted with 1 ml of washing buffer in separate collection tubes by removing the columns from the separator and by firmly pushing the plunger into the columns.

3.11 RNA EXTRACTION AND QUANTITATIVE PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and 0,8 ug of extracted RNA was reversed transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad) according to the manufacturers's instruction. Realtime PCR was performed with 10 ng of cDNA as template, 0,2 uM primers and 1X iQ Custom SYBR Green Supermix (Biorad) in a 20 ul final volume using 7900 HT Fast Real time PCR System instrument (Applied Biosystems).

The following primers were used to amplify target genes: human alkaline phosphatase (ALP) forward 5'-CGCACGGAACTCCTGACC-3' and reverse 5'-GCCACCACCACCATCTCG-3', human osteopontin (SPP1) F5'-TGCTACAGACGAGGACATCA-3' and

R5'-GTCATCCAGCTGACTCGTTT-3', human runt-related transcription factor 2 (RUNX2)

F5'-CACCATGTCAGCAAAACTTCTT-3' and R5'-TCACGTCGCTCATTTTGC-3', R5'-CGGACTGTGTCTGCTGTGTT-3'. Human tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) F5'-GATCCCCAATGCTTCACAAG-3' and

R5'-TGCTTGTTGTGACTGATCGAC-3' primers were used to amplify reference gene. The following amplification program was used: initial denaturation at 95°C for 3 min, followed by 40 cycles of 5 sec each at 95°C and 30 sec at 56°C. Relative quantification of target genes was performed in triplicate and was calculated by the equation $2^{-\Delta\Delta Ct}$

3.12 HPTLC-MASS SPECTROMETRY

After development, HPTLC plates were placed into the ImagePrep device (Bruker Daltonics) and matrix layer was added by repeating three identical phases with 8 spray cycles, followed by 30 seconds incubation time and 30 seconds of drying time (four phases for neutral GSLs). Subsequently, HPTLC plates were placed on the MTP TLC adapter (Bruker Daltonics) and transferred to MALDI mass spectrometer.

Spectra were acquired in reflector negative modality depending by using an Ultraflex III ToF/ToF mass spectrometer equipped with Smartbeam laser (frequency of 100 Hz, Bruker Daltonics) and FlexControl software v. 3.3 (Bruker Daltonics). Spectrometer settings were ion source 1, 20 kV; ion source 2, 17.2 kV; lens, 7.4 kV; reflector 21 kV; reflector 2, 11 kV; mass suppression up to m/z 800; pulsed ion extraction, 200 ns; detector gain voltage, 1552 V; electronic gain, 100 mV/full scale.

SurveyViewer software v.1.1 (Bruker Daltonics) was employed.. Spectra of interest were then analyzed by FlexAnalysis software v. 3.3 (Bruker Daltonics). The attribution of spectra to gangliosides was made by the LIPID MAPS structure database (LMSD, Sud M., Fahy E., Cotter D., Brown A., Dennis E.,

Glass C., Murphy R., Raetz C., Russell D., and Subramaniam S., *Nucleic Acids Research* **35**, D527-32 (2006)) with a GSLs MS precursor ion analysis tool (LIPID MAPS online tools for lipid research. Fahy E, Sud M, Cotter D and Subramaniam S. *Nucleic Acids Research* **35**, W606-12 (2007)).

3.13 ALKALINE PHOSPHATASE ACTIVITY QUANTIFICATION

Alkaline Phosphatase was quantified in GD1a positive and GD1a negative BMSCs differentiated to osteoblasts and in non-differentiated BMSCs as control using Quantitative Alkaline Phosphatase ES Characterization kit (Millipore) according to the manufacturer's instructions. Cells were washed and harvested in PBS by scraping, collected in 1X Wash Solution for counting and centrifugated at 1200 rpm for 6 minutes. 45000 cells for each sample were resuspended in 150 ul of p-NPP Buffer and tranferred to 3 wells of a 96-well plate (15000 cells/well). The enzymatic reaction was performed adding 50 ul of 2X p-NPP Substrate Solution to each sample and incubating for 20 minutes at RT in the dark. At the end of the incubation time, reaction was stopped by adding 50 ul of Reaction Stop Solution. The amount of p-nitrophenol, a yellow colored by-product of the catalytic reaction, was mesured reading the absorbance at 405 nm using Victor^3 plate reader (Perkin Elmer).

CHAPTER 4 - RESULTS

4.1 BMSCs CHARACTERIZATION

Human MSCs extracted from bone marrow of different donors showed the characteristic features of spindle shape and plastic adherence. The culture-expanded cells were capable of *in vitro* differentiation to osteoblasts as demonstrated by alizarin red staining (fig. 1 A), to adipocyte as demonstrated by oil red staining (fig 1 B), to smooth muscle cells as demonstrated by smooth muscle α-actin immunofluorescence staining (fig. 1 C) and to chondroblasts as demonstrated by alcian blu staining. (fig. 1D) Cytofluorimetric analysis was performed to test the expression of currently used MSCs markers and the absence of hematopoietic and endothelial antigens. BMSCs resulted to be $CD73^+$, $CD90^+$, $CD105^+$, $CD3^-$, $CD11b^-$, $CD19^-$, $CD34^-$, $CD45^-$ and $HLA\text{-}DR^-$ as expected. (fig. 1E)

Figure 1

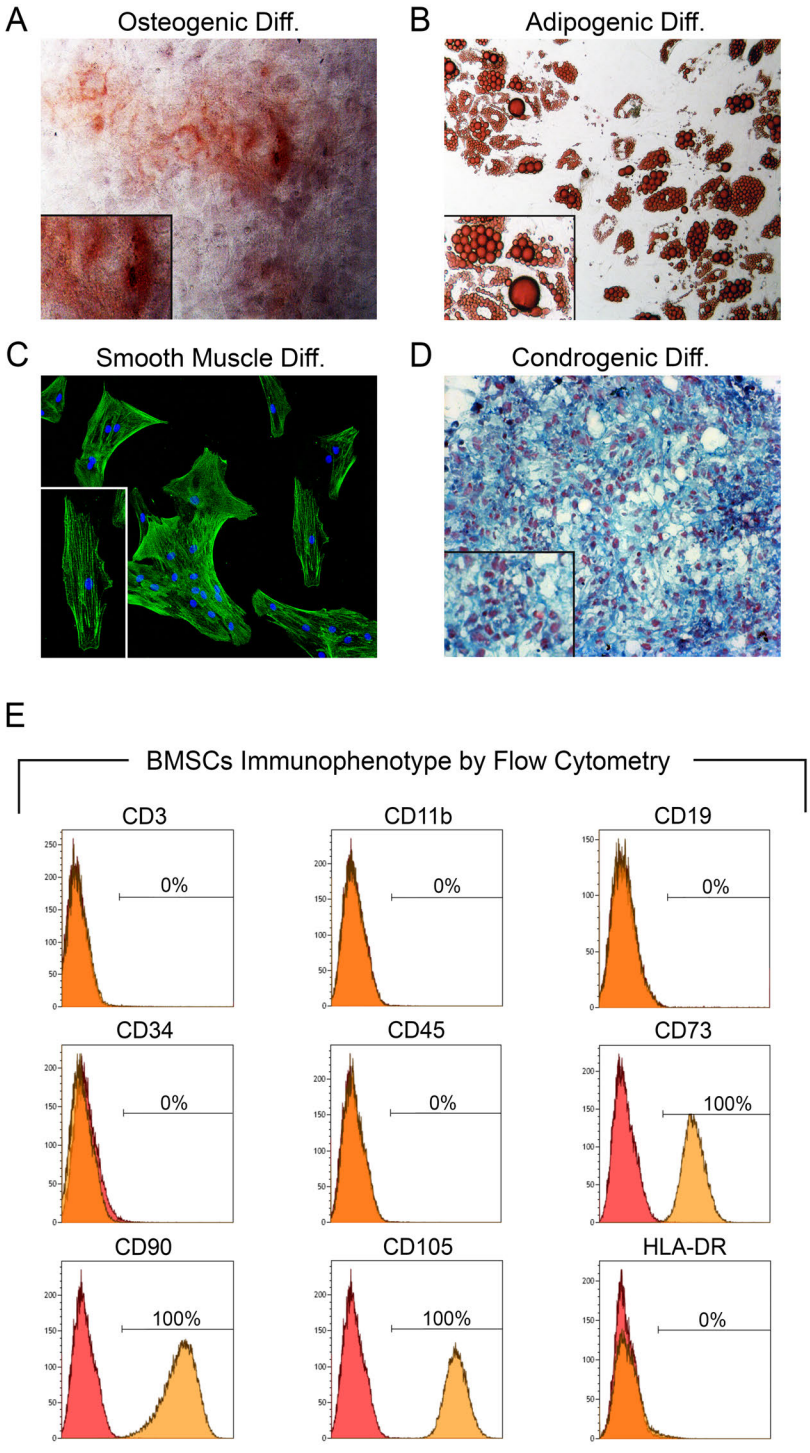

Figure 1 (A) Alizarin red stain, (B) oil red o stain, (C) immunofluorescence staining of smooth muscle α-actin using FITC-conjugated anti-human α-actin antibody and (D) alcian Blue stain. (E) Flow cytometry analysis of antigens commonly used to define BMSCs: CD73, CD90, CD105, CD3, CD11b, CD19, CD34, CD45 and HLA-DR (the peaks of specific antigens are shown in yellow while the peaks of respective isotype controls are shown in red).

4.2 ANALYSIS OF SL PATTERN

SL pattern of BMSCs was analyzed by metabolic labeling of cell sphingolipids with [1-^{3}H]Sphingosine, as described in the Methods. Cells were collected and, after lipids extraction, the organic and aqueous phase radiolabeled sphingolipids were separated by HPTLC and quantified by radiochromatoscanning (fig. 2 A and C). In the *organic* phase, we found that BMSCs expressed mainly SM, Gb4, and Gb3As, as shown in the percentage distribution graph (fig. 2B). On the other hand, SLs found in the aqueous phase were mainly a-series gangliosides, GM3 being the most abundant, and the b-series ganglioside GD3 (fig. 2 D). Surprisingly, ganglioside GD2, that was proposed by Martinez et al. as a marker for BMSCs characterization and isolation, could not be detected in any of the sample analyzed. Results obtained from radiolabeled SLs analyses confirmed the results obtained by the less sensitive methodology of HPTLC separation of BMSCs endogenous non-radiolabeled SLs (fig. 2A and C, first lanes).

Figure 2

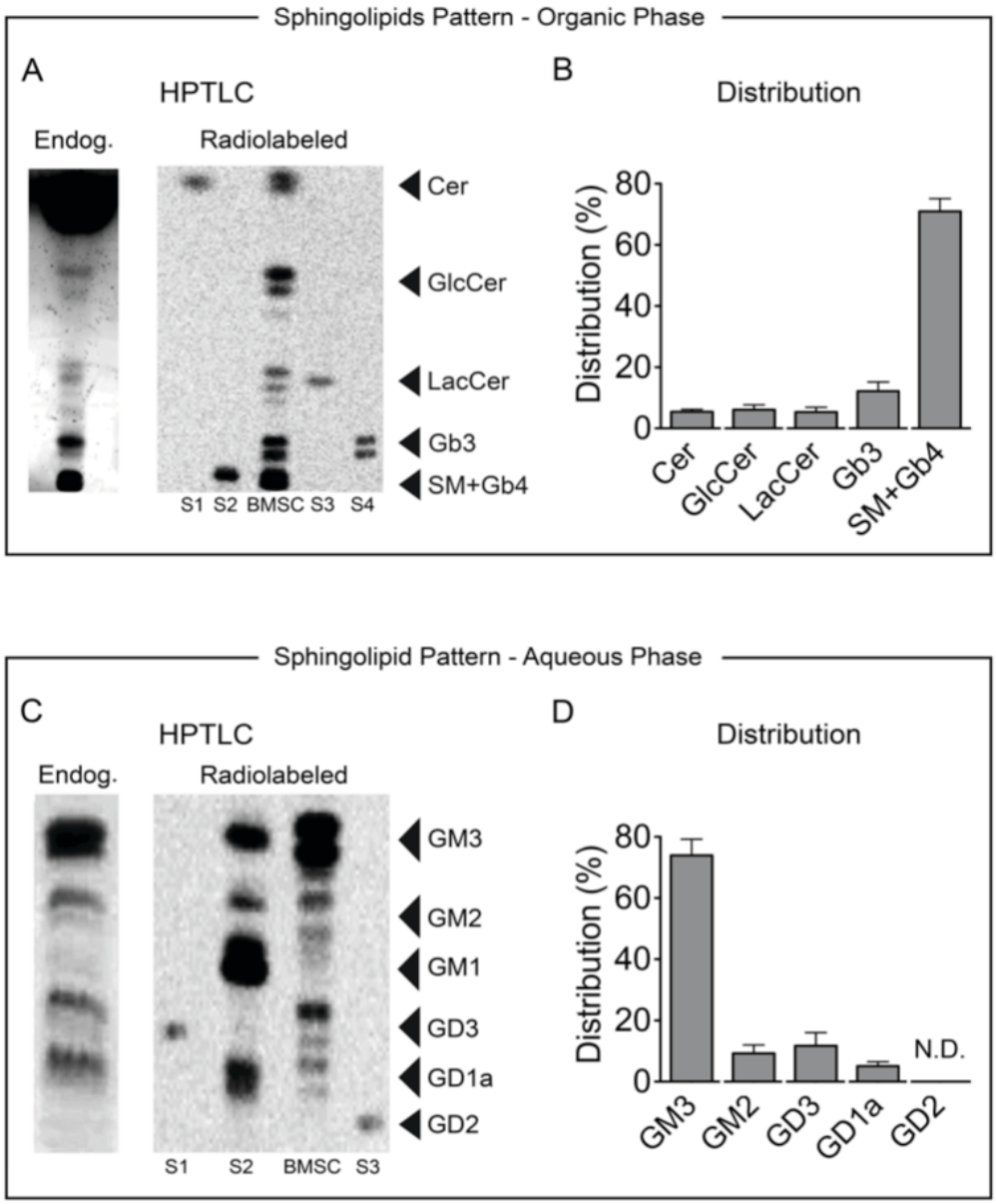

Figure 2 SL pattern of BMSCs. Cells were metabolically labelled with [3-^{3}H]sphingosine and after lipid extraction organic and aqueous phase SLs were separated by HPTLC.
Top panel: (A) HPTLC separation of BMSCs organic phase SLs developed in 110:40:6 (v/v) chloroform/methanol/water solvent. Radiochromatoscanning images of endogenous GSLs visualized using Anisaldeide solution and radiolebeled GSLs visualized with a Beta-Imager 2000. (B) Percentage distribution of radiolebeled GSLs.
Bottom panel: HPTLC separation of BMSCs aqueous phase gangliosides developed in 60:40:9 (v/v) chloroform/methanol/0,2% $CaCl_{2.}$ solvent. Radiochromatoscanning images of endogenous gangliosides visualised using Earlich's reagent and radioalebeld gangliosides visualised with a Beta-Imager 2000. (D) Percentage distribution of radioalebeled gangliosides The radioactivity associated with individual lipids was determined with the specific β–Vision software. Data are means ±SD of five different experiments..

4.3 ANALYSIS OF GD2 EXPRESSION BY HPTLC-MASS SPECTROMETRY

To further investigate the presence of ganglioside GD2 in BMSCs, cell sphingolipids were analyzed also by a combined HPTLC-mass spectrometry approach. Mass spectra were acquired in negative reflection mode by direct coupling of the HPTLC of endogenous non-radiolabeled SLs with a MALDI mass spectrometer. Figure 3 shows representative radiochromatoscanning images of HPTLC separation of (A) BMSCs radiolabeled gangliosides, (C) BMSCs radiolabeled gangliosides spiked with GD2 standard and (E) GD2 standard alone as positive control. No signal corresponding to GD2 was detected in any of the representative mass spectra obtained from the analysis of BMSCs gangliosides (fig. 3 B). On the contrary, signals corresponding to GD2 were observed in mass spectra obtained from the analysis of gangliosides spiked with GD2 standard. In particular, peaks at 1673,92 m/z and at 1701,92 m/z correspond to GD2 d18:1, C18:0 $[M-H]^-$ and C20:0 $[M-H]^-$, respectively, and peaks at 1694,92 m/z and 1722,92 correspond to C18:0 $[M+Na-2H]^-$ and to C20:0 $[M+Na-2H]^-$ (Fig. 3D). Mass spectra obtained from the analysis of GD2 standard alone showed signals corresponding to GD2 as expected. In particular, peaks at 1673,92 m/z and at 1701,92 m/z correspond to GD2 d18:1, C18:0 $[M-H]^-$ and C20:0 $[M-H]^-$, respectively, and peaks at 1694,92 m/z and 1722,92 correspond to C18:0 $[M+Na-2H]^-$ and to C20:0 $[M+Na-2H]^-$ (Fig. 3F). These data confirmed results obtained with HPTLC analysis demonstrating that ganglioside GD2 was not expressed in BMSCs.

Figure 3

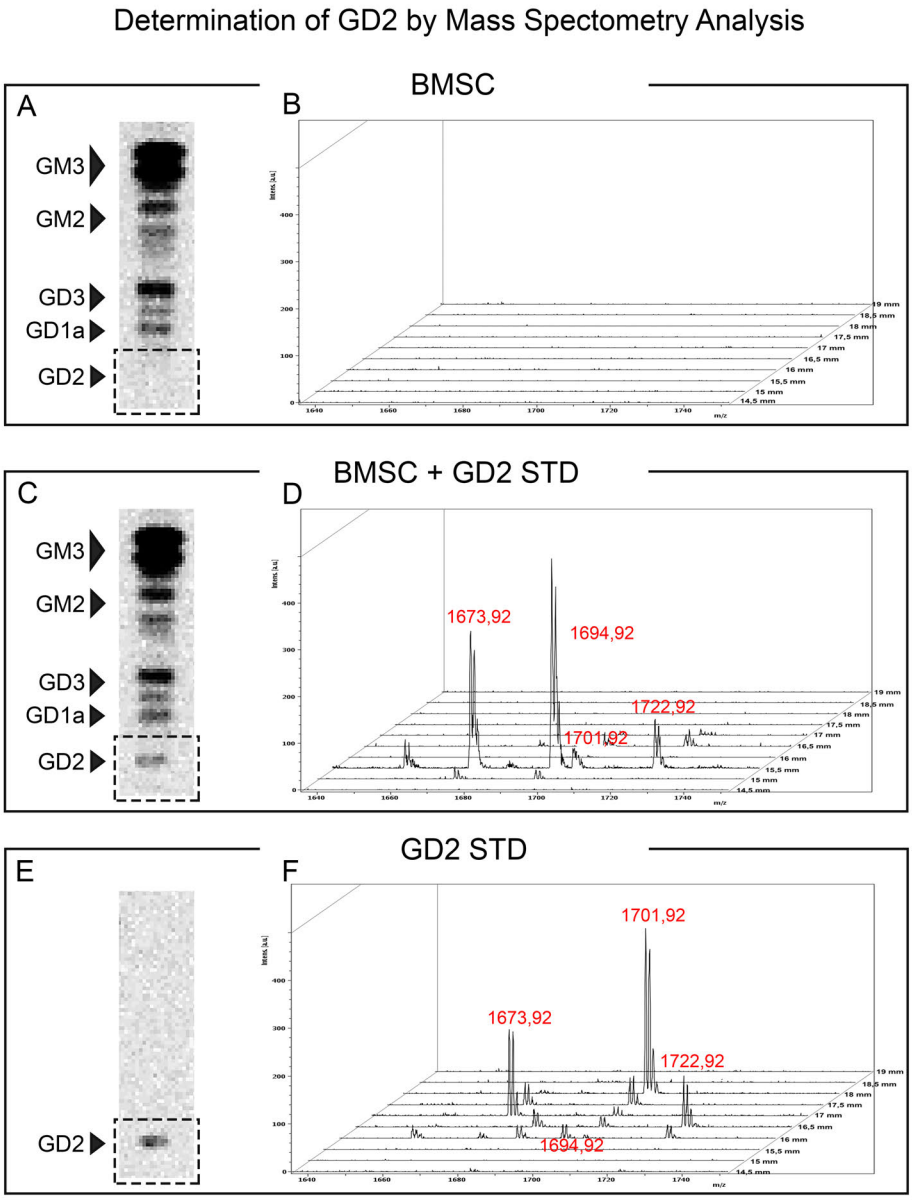

Figure 3 HPTLC separation and mass spectrometry.
(A) Representative radiochromatoscanning image of BMSCs radiolabeled gangliosides. The close up of the image shows the area corresponding to GD2 standard band position analyzed by mass spectrometer. (B) No signals were detected in spectra of the acquired region, from 14,5 to 19 mm with a 0,5 mm gap. (C) Representative radiochromatoscanning image of BMSCs radiolabeled gangliosides spiked with GD2 standard. The close up of the area of GD2 standard shows mass spectra of the ganglioside. (D) Peaks at 1673,92 m/z and at 1701,92 m/z correspond to GD2 d18:1, C18:0 $[M-H]^-$ and C20:0 $[M-H]^-$, respectively, and peaks at1694,92 m/z and 1722,92 correspond to C18:0 $[M+Na-2H]^-$ and to C20:0 $[M+Na-2H]^-$. (E) Representative radiochromatoscanning image of GD2 standard alone. The close up of the area of the region corresponding to GD2 standard shows the mass spectra of the ganglioside. (F) Peaks at 1673,92 m/z and at 1701,92 m/z correspond to GD2 d18:1, C18:0 $[M-H]^-$ and C20:0 $[M-H]^-$, respectively, and peaks at1694,92 m/z and 1722,92 correspond to C18:0 $[M+Na-2H]^-$ and to C20:0 $[M+Na-2H]^-$.

4.4 ANALYSIS OF GD2 EXPRESSION BY IMMUNOLOGICAL TECHNIQUES

Successively, we investigated whether discrepancies between our results (showing an undetectable level of ganglioside GD2 in BMSCs) and the published data (suggesting GD2 as a new BMSCs marker) were caused by the different analytical methodology used. In particular, as antibodies are often unspecific in recognizing small non-proteic epitopes, we tested whether we could observe GD2-positivity by immunohistochemistry in the same samples, which we previously found negative for GD2 expression. Therefore, we analyzed BMSCs by cytofluorimetry, using the same anti-GD2 antibody used by Martinez et al. Surprisingly, under these conditions, we observed that 19,5% ± 5% of all BMSC samples analyzed were positive for GD2. (fig. 4 A) Thus, to test whether the observed results could be due to an unspecific binding of the GD2-antibody, we tried to enrich the GD2-positive population by sorting cells with immunobeads carrying the anti-GD2 antibody. The so called "positive" and "negative" fractions were collected and analyzed by cytofluorimetry, to test whether the enrichment step was successful. Interestingly, the positive fraction was 70,5% ± 10,8% positive by FACS analysis, while the negative fraction was only 9,7% ± 4,5%, confirming that the antibody was specifically binding to a fraction of BMSCs (Fig. 4 C and D). Nonetheless, these results do not clarify if the recognized epitope is really GD2 or the antibody is binding some other molecule. Therefore, we analyzed the sphingolipid pattern of both positive and negative cell fractions by metabolic labeling of cell sphingolipids with [1-^{3}H]Sphingosine followed by HPTLC separation and analysis with radiochromatoscanning. We found almost

superimposable sphingolipid patterns in the two cell populations. In particular, GD2 could not be detected in the negative but also in the positive cell fraction, suggesting that anti-GD2 antibody possibly recognized other targets (Fig. 4 E and F). These results may support the notion that GD2 antibody can also cross-react with other sialo-glycoconjugates, which have yet to be identified.

Figure 4

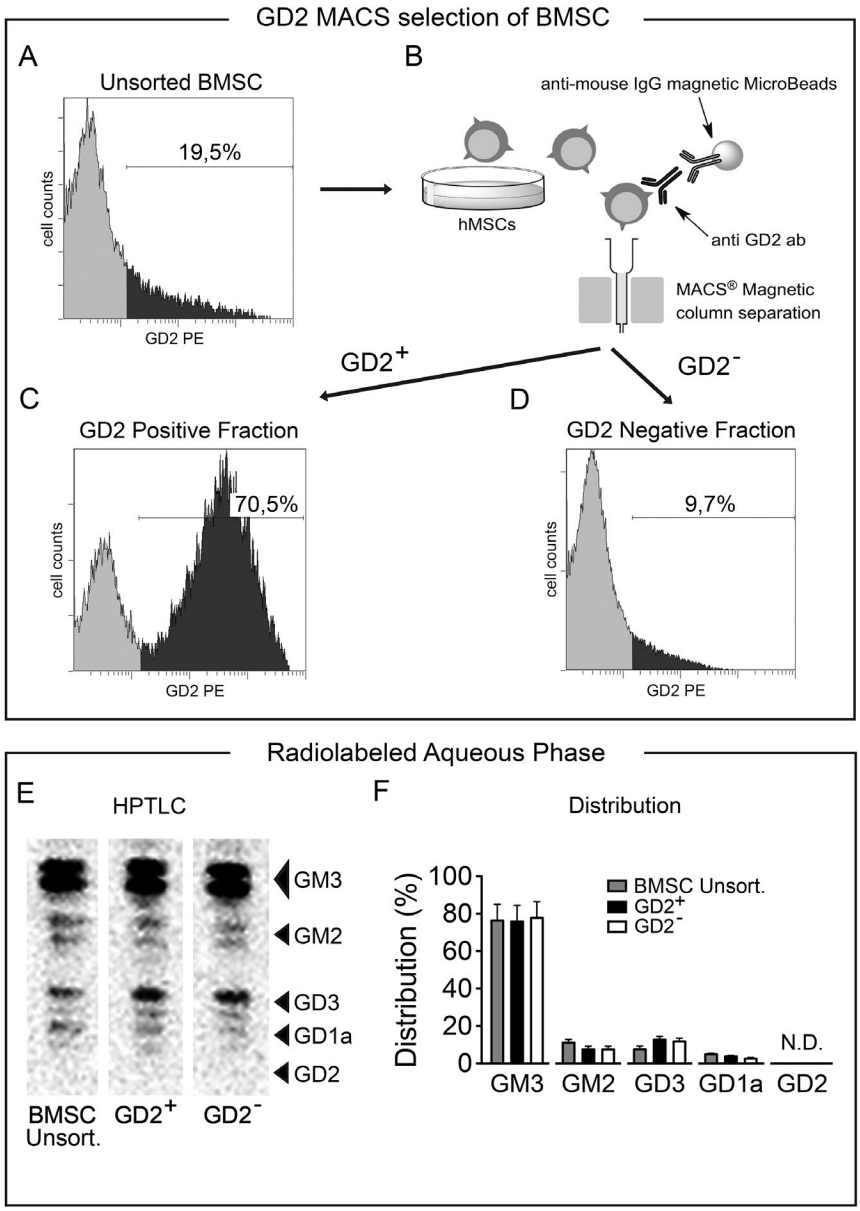

Figure 4 Immunoselection of BMSCs for ganglioside GD2.
(A) Flow cytometry analysis of GD2 expression in BMSCs.
(B) Schematic image of immunobeads separation protocol (Miltenyi Biotech). (C-D) Flow cytometry analysis of GD2 expression in GD2 positive (C) and GD2 negative (D) cell fractions obtained after immuobeads separation. (E) Radiochromatoscanning image of HPTLC separation of radiolabelled gangliosides: *lane 1*: unsorted BMSCs gangliosides, *lane 2*: GD2 positive BMSCs gangliosides, *lane 3*: GD2 negative BMSCs gangliosides.
(F) Percentage distribution of radiolabelled gangliosides. Data are means ±SD of three different experiments.

4.5 ANALYSIS OF SL PATTERN MODULATION DURING BMSCs DIFFERENTIATION

The SL pattern of BMSCs was then studied during stem cell differentiation, to assess a possible involvement of SLs in the process. Thus, the initial sphingolipid pattern of BMSCs isolated from five different donors was analyzed by metabolic labeling of cell sphingolipids with [1-^{3}H]sphingosine, as previously described (fig. 6 A). We found small variability in gangliosides distribution among BMSCs from different donors (fig. 6 B). Then, cell were induced to differentiate and acquire the osteoblastic phenotype, by culturing cells in the appropriate differentiation medium, as described in the Methods section. The differentiation process is usually completed within 2-3 weeks from induction. Nonetheless, in order to investigate the possible involvement of some particular SLs in the induction of the differentiation process, we focused our attention on the early phases of the process. Therefore, we analyzed the SL pattern modifications at day 5 from differentiation, where some crucial osteogenic genes are known to be already activated. In fact, ALP gene expression was about 9,8-fold higher in BMSCs induced to osteogenic differentiation respect to undifferentiated cells (fig. 5).

Metabolic radiolabelling with [3-^{3}H]sphingosine revealed a significant decrease (23% reduction) of GM3 content at day 5 of differentiation, whereas a-series gangliosides markedly increased, in particular GM2, GM1 and GD1a (54,2%, 50,7% and 72,3% increase, respectively, Figure 6 D). On the contrary, we did not observe a significant change in ganglioside GD3 content (Fig 6 D) as well

as in organic phase SLs between differentiated BMSCs and control undifferentiated cells (fig. 6 E and F)

Figure 5

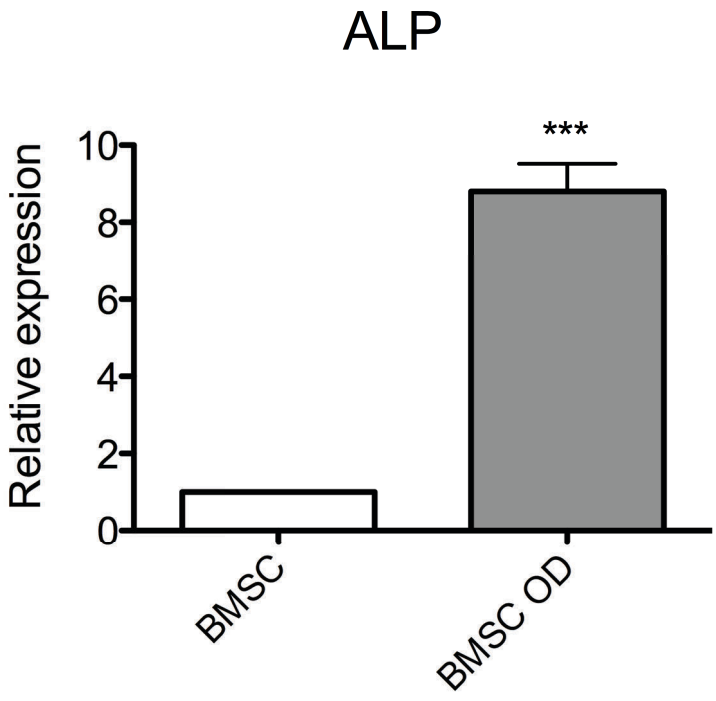

Figure 5 Realtime PCR analysis of ALP in BMSCs induced to differentiate toward osteoblasts for 5 days. Undifferentiated BMSCs were used as control. Data shown are means ± SD ot three different experiments. Statistical differences were determined by 1- way Anova: *** $P<0.0001$ compared to respective controls.

Figure 6

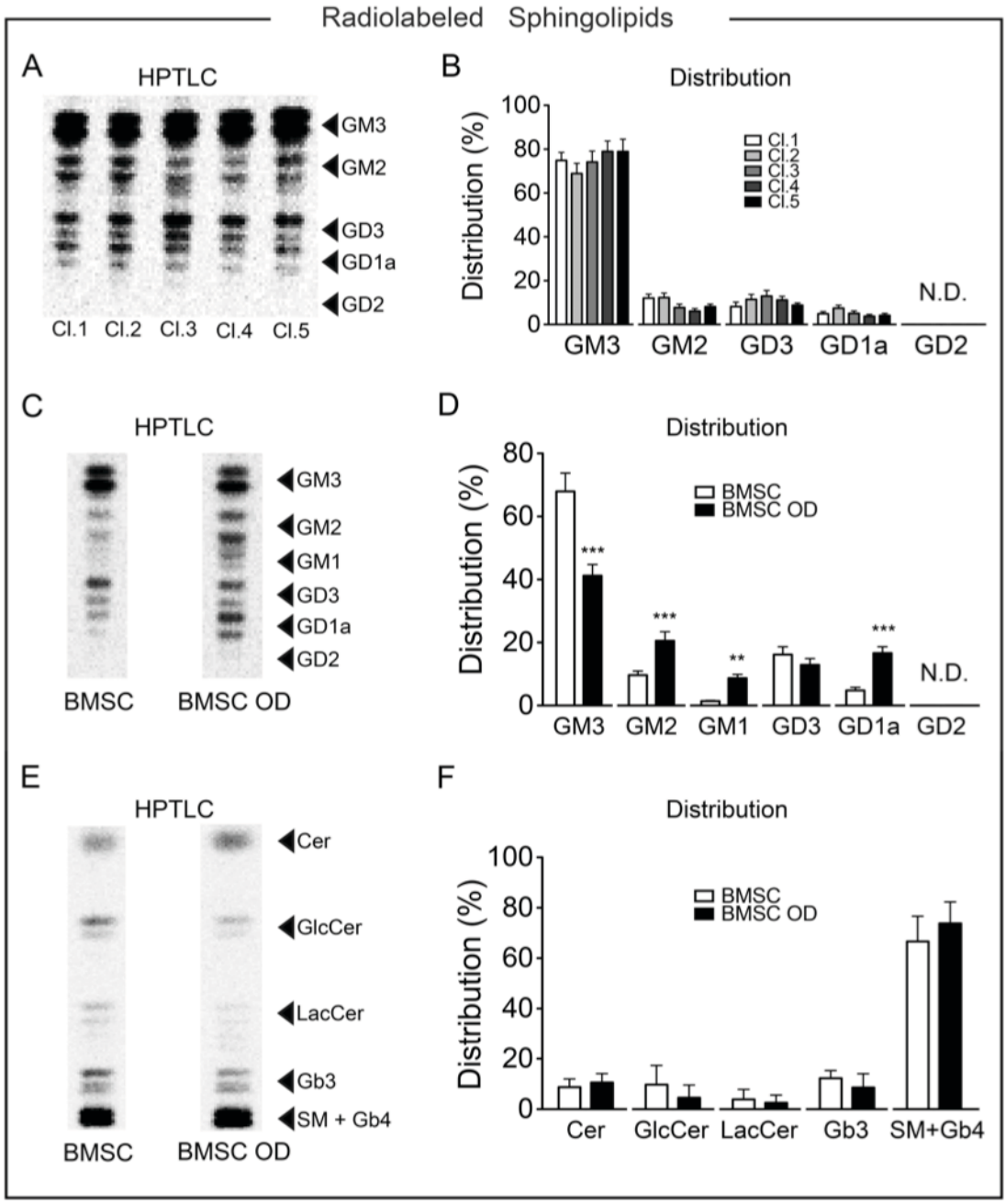

Figure 6 (A) Radiochromatoscanning image of HPTLC separation of radiolabeled gangliosides extracted from BMSCs of different donors. (B) Percentage distribution of radiolabeled gangliosides. Data are means ±SD of three different experiments. (C) Radiochromatoscanning image of HPTLC separation of aqueous phase radiolebeled gangliosides extracted from BMSCs differentiated toward osteoblasts. *Lane 1*: gangliosides of control undifferentiated BMSCs, *lane 2*: gangliosides of BMSCs differentiating toward osteoblasts. (D) Percentage distribution of radiolabeled gangliosides. (E) Radiochromatoscanning image of HPTLC separation of radiolebeled organic phase SLs extracted from BMSCs differentiated toward osteoblasts. *Lane 1*: SLs of control undifferentiated BMSCs, *lane 2*: SLs of BMSCs differentiating toward osteoblasts Data are means ±SD of three different experiments, statistical differences were determined by 1- way Anova: **P<0.001 and *** P<0.0001 compared to control.

4.6 BMSCs ENRICHMENT IN GANGLIOSIDE GD1a

As ganglioside GD1a expression greatly increased during osteogenesis, we tested if this ganglioside could represent a new early marker of stem cell commitment toward bone differentiation. Therefore, we checked if we could enrich the initial BMSCs population in GD1a using the opportune antibody. Based on the previous unsuccessful results with GD2, we first needed to assess if the anti-GD1a antibody could specifically bind GD1. Therefore, we first analyzed cells by cytofluorimetry and found that only 25% ± 6% of the cell population was positive for GD1a. Therefore, we envisioned the possibility of selecting, or at least enriching, this GD1a positive sub-population using immunobeads sorting, as previously described for GD2. After immunobeads sorting, results confirmed that we could indeed obtain two populations, one highly enriched in GD1a (94% ± 9% positive), and one putatively GD1a negative (13 ± 4,5) (fig. 7 A). Differently from our previous attempts with GD2, metabolic radiolabelling with [3-^{3}H]sphingosine (fig. 7 B) revealed that the two cell fractions (GD1a positive and negative) possessed different expression of GD1a. In fact, in the positive fraction GD1a percentual content was 5,4 whereas in the negative one only 0,98. (Fig. 7 C)

4.7 ANALYSIS OF OSTEOGENIC POTENTIAL OF GD1a POSITIVE AND GD1a NEGATIVE BMSCs

To test the hypothesis that GD1a could represent a marker of BMSCs initial commitment to the osteogenic phenotype, we analyzed gene expression of

osteogenic markers genes ALP, osteopontin and RUNX2 in GD1a positive and GD1a negative cells, and compared the results to unsorted BMSCs. Analysis of gene expression by qPCR revealed that GD1a positive BMSCs possessed significantly higher mRNA levels of all osteogenic markers analyzed as compared to the GD1a negative fraction, as shown in Fig. 7 D, E, F. In particular in GD1a positive BMSCs ALP, osteopontin and RUNX2 gene expressions were 2,3-fold, 2,6-fold and 0,5-fold increased, respectively, in comparison to GD1a negative BMSCs.

Then, we tested whether the initial osteogenic commitment of GD1a-positive BMSCs could affect the differentiation process. Therefore, GD1a-positive and –negative cell fractions were induced to differentiate toward osteoblasts, as previously described. At the end of the differentiation process, which was carried out for 3 weeks, we checked the expression of osteogenic markers ALP, osteopontin and RUNX2. Undifferentiated, unsorted BMSCs were used as controls. Results confirmed a significantly higher expression of all osteogenic markers in differentiated osteoblasts originated from GD1a-positive cells (Fig. 6 G,H,I). In particular, we registered a 1,2-fold increase of ALP, 2,4-fold increase of osteopontin and 1,2-fold increase of RUNX2 in differentiated GD1a positive cells respect to differentiated negative ones. To further confirm that GD1a-positivity enhanced the induced osteogenesis, the enzymatic activity of ALP was also measured at the end of the differentiation process. We found that ALP enzymatic activity was almost 3-fold higher in osteoblasts originated from GD1a-positive BMSCs as compared to those obtained from GD1a negative ones (Fig. 6 J).

Figure 7

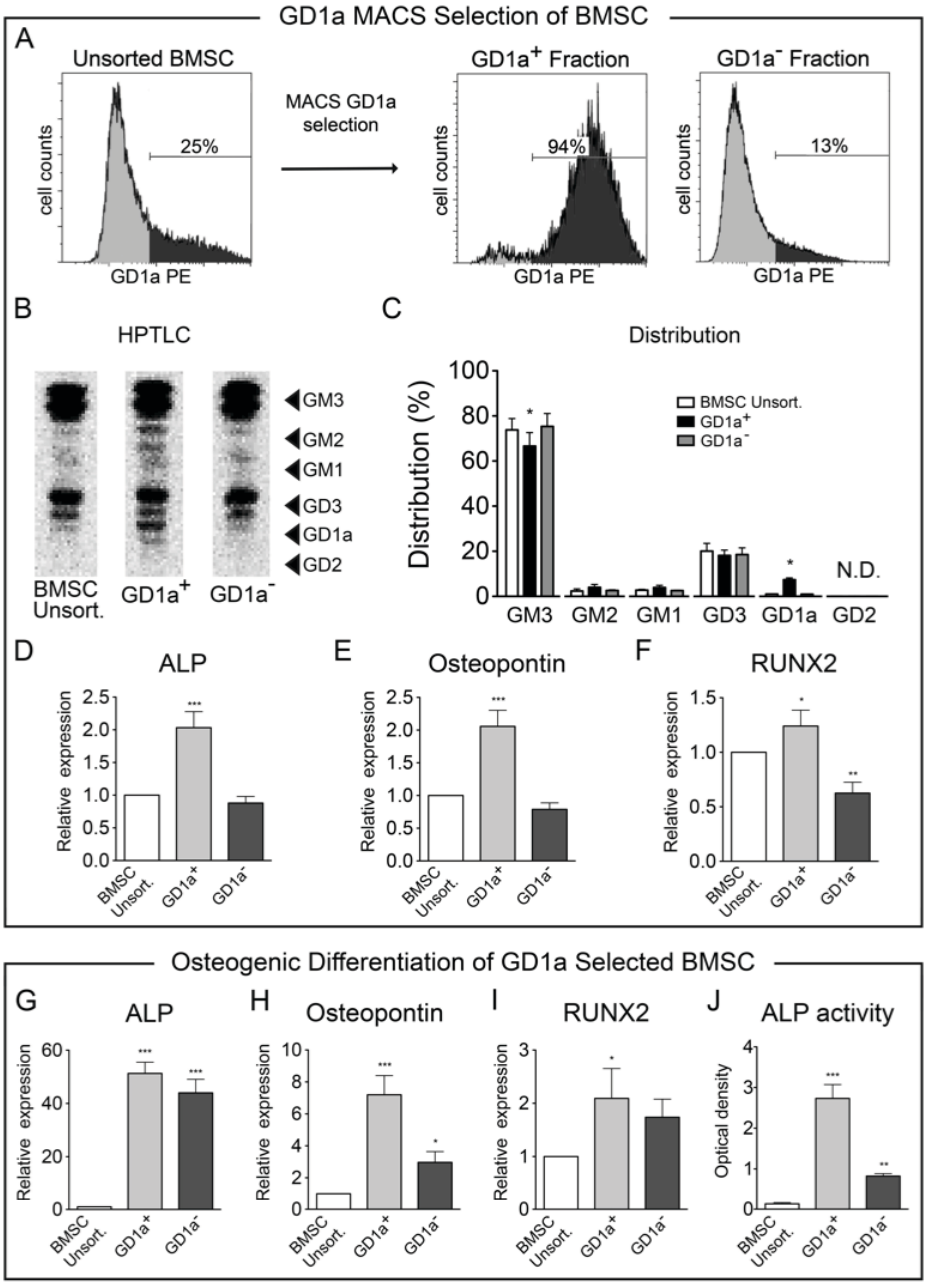

Figure 7 Immunobeads separation of BMSCs for ganglioside GD1a.
(A) Flow cytometry analysis of GD1a expression in BMSCs and in GD1a negative and GD1a positive cell fractions obtained after immunoselection. (B) Radiochromatoscanning image of HPTLC separation of radioalebeld ganglioside: *lane 1*: unsorted BMSCs gangliosides, *lane 2*: GD1a positive BMSCs gangliosides, *lane 3*: GD1a negative BMSCs gangliosides. (C) Percentage distribution of radiolebeled gangliosides. (D-F) Realtime PCR analysis of ALP, osteopontin and RUNX2 gene expression in GD1a positive and negative BMSCs compared to unsorted undifferentiated BMSCs. (G-I) Realtime PCR analysis of ALP, osteopontin and RUNX2 gene expression in GD1a positive and negative BMSCs differentiated toward osteoblasts compared to unsorted undifferentiated BMSCs. (J) Graph showing ALP activity in GD1a positive and negative BMSCs differentiated toward osteoblasts and in unsorted undifferentiated BMSCs as control. Data shown are means ±SD of three different experiments, statistical differences were determined by 1- way Anova: *P<0.05 , **P<0.001 and *** P<0.0001 compared to respective controls.

CHAPTER 5 - DISCUSSION

Despite the recent advances in the stem cell field, many issues of stem cells biology and their clinical use remain unresolved. MSCs derived from different tissues were extensively investigated in the last few years. However, there is still a lack of specific cell markers for the identification of these cells.

In fact, MSCs are currently defined by a combination of physical, phenotypic and functional properties and novel cell- surface markers are therefore needed for their quick characterization and isolation from tissues. In this work, SLs, a class of lipids principally localized in plasma membrane and involved in many cell signaling pathways, were investigated as possible cell surface markers for BMSCs identification.

We first characterized MSCs isolated from bone marrow of different donors through cytofluorimetric analysis. As expected, these cells expressed the typical MSCs markers CD90, CD105 and CD73 and lack the expression of hematopoietic and endothelial antigens. Moreover, cells were able to differentiate toward osteoblasts, adipocytes, chondroblasts and smooth muscle cells demonstrating the tipical multi-lineage differentiation ability of MSCs.

The analysis of SL pattern was performed according to metabolic labeling of cells SLs with [3-^{3}H]sphingosine, an established methodology which has been used and perfected in our laboratory in the past several years. Radiolabeled SLs were extracted and partitioned into an organic and an aqueous phases,

and then subjected to chromatography on HPTLC plates. Regarding the organic phase SLs, BMSCs expressed principally SM, an important element of all plasma membranes, and the globo-serie SLs Gb4 and Gb3. Among aqueous phase SLs, we found that BMSCs expressed mainly gangliosides of a-series, such as GM3, GM2 and GD1a, and b-series ganglioside GD3. Our findings are in contrast with those published by Martinez and colleagues,[180] who reported the expression of the b-series ganglioside GD2 in BMSCs, which they proposed as a novel surface marker for BMSCs identification and isolation.

Therefore, to better investigate GD2 content of BMSCs, we developed a new combined HPTLC-MALDI mass spectrometry approach, in collaboration with some colleagues from our Department. This method allowed us to directly obtain the mass spectra of cells endogenous gangliosides separated by HPTLC. Results confirmed the sphingolipid pattern that we could observe using metabolic labeling, and GD2 ganglioside could not be detected in any of the analyzed samples.

At this point, we decided to investigate whether discrepancies between our and published data on GD2 expression in BMSCs were caused by the different analytical methodology used. Indeed, Martinez and colleagues demonstrated GD2 expression in BMSCs only through immunological techniques, using an anti-GD2 antibody. Unfortunately, although widely used, antibodies against gangliosides are not always specific, as they may recognize saccharide moieties present on other glyococonjugates or glycoproteins. Along this line, Apostolski and colleagues reported that a subset of human anti-GM1

antibodies cross-react with Gal(beta 1-3)GalNAc bearing glycoproteins in peripheral nerve and spinal cord.[193] Moreover, Thomas and colleagues demonstrated that IgM monoclonal antibodies binding ganglioside GD1b, recognize also several glycoproteins in the central and peripheral nervous system[194]. Therefore, as antibodies are often unspecific in recognizing non-proteic molecules, we tested if we could observe GD2-positivity in BMSCs through cytofluorimetric analysis, using the same anti-GD2 antibody used in the published work mentioned above. Actually, we found that a subpopulation of BMSCs was linked by this antibody. Moreover, we could separate this subpopulation using immunobeads linked with anti-GD2 antibody. The cell fraction obtained after separation resulted GD2 positive according to cytofluorimetric analysis. Therefore, we checked by biochemical analysis whether this cell population really expressed ganglioside GD2. Results of HPTLC separation showed that GD2 positive BMSCs had the same ganglioside pattern as GD2 negative ones. In particular, GD2 was not detectable in any of the analyzed fractions, suggesting that in these cells, the anti-GD2 antibody possibly recognized other targets than GD2.

These results underlined that antibodies should always be used with great caution, and that multiple analytical approaches should be always used, especially when analyzing small non-proteic biomolecules. Therefore, we tend to exclude that ganglioside GD2 could be elected as specific BMSCs marker, although it would be useful to analyze cells isolated by other labs.

Nonetheless, as SLs are key molecules involved in cell proliferation and differentiation, we tested if other sphingolipids, besides GD2, could be used as

stem cell markers and/or could be involved in BMSC differentiation. Therefore, in the second part of this thesis work, we focused our attention on SLs role in cell differentiation process. MSCs are known to possess the potential to differentiate into many different tissues. However, one of the main problems linked to the use of these cells in regenerative medicine is that the number of cells that differentiate into the desired phenotype, upon opportune stimulation, is often too low. Moreover, the mechanism by which they repair damaged tissues *in vivo* is still poorly understood and we are not able to drive differentiation and cell behavior in a controlled fashion. Published data revealed that SL pattern of adult stem cells significantly changes with cell differentiation. In particular, it was demonstrated that in human ADMSCs GD1a content increases after 5 days of differentiation toward osteoblasts. (lee) Moreover, in MSCs from dental pulp, it was observed an increase of ganglioside GD1a together with a decrease of ganglioside GM3 during differentiation toward osteoblasts.[185]

Based on these premises, we wanted to investigate whether SLs content of BMSCs was modulated during differentiation toward osteoblasts and whether its modulation was directly linked to the differentiation process. We initially observed that ganglioside pattern of BMSCs strongly changed during the differentiation. In particular, we registered a strong increase of a-series gangliosides GM2 and GD1a already after 5 days of osteogenic induction. To test if GD1a could be implicated in osteoblasts differentiation, we first analyzed the expression of osteogenic markers genes ALP, osteopontin and RUNX2, in GD1a positive and GD1a negative BMSCs. ALP gene is not specifically

expressed by osteoblasts. However, its expression notably increases after osteogenic induction and it is considered, together with osteopontin, an early marker of osteogenic differentiation. Results of quantitative PCR showed that the expression of ALP and osteopontin was significantly higher in GD1a positive cells than in GD1a negative ones. Moreover, the expression of RUNX2, a transcription factor essential for the differentiation toward osteoblasts, resulted to be increased in GD1a positive cells compared to GD1a negative ones. Consequently, we decided to evaluate the osteogenic potential of GD1a positive as compared to that of GD1a-negative BMSCs. Results indicated that the subpopulation of BMSCs expressing GD1a had a higher osteogenic potential as compared to GD1a negative BMSCs. This was possibly caused by an initial osteogenic commitment of GD1a-positive BMSCs, as they possessed higher basal level of osteogenic markers, as compared to the GD1a-negative cell fraction. Therefore, these data suggest that GD1a positive BMSCs may represent a sub-population of mesenchymal progenitor cells which are already partially committed to osteogenic differentiation. On the contrary, GD1a negative BMSCs could represent a more undifferentiated subpopulation of MSCs. Therefore, we are currently investigating whether GD1a-negative BMSCs, beeing a more undifferentiated cell population, posses a higher plasticity due to less commitment. Moreover, the role of the other SLs that we found modulated upon BMSCs differentiation (GM3, GM2 and GM1) is also under further investigation.

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