Roles of gangliosides in the differentiation of mouse pluripotent stem cells to neural stem cells and neural cells (Review)

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Received June 14, 2016; Accepted April 12, 2017

DOI: 10.3892/mmr.2017.6719

Abstract. Glycosphingolipids are important components of the outer layer of the plasma membrane in the majority of eukaryotic cells. Specifically, gangliosides are sialic acid-containing glycosphingolipids that participate in cell-cell recognition, adhesion, proliferation, differentiation and signal transduction, and are integral components of cell surface microdomains and lipid rafts. Stem cells are defined functionally as cells that have the capacity to self-renewal and differentiate to generate various cell types. Due to different synthesis patterns and locations of gangliosides, they have been used as molecular markers of stem cells. The current review describes the presence of gangliosides in various types of mouse stem cells, including pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells) and neural stem cells, and the functional roles of gangliosides in various processes, including cell proliferation and neural differentiation. Thus, this review will aid the understanding of gangliosides patterns and functions in mouse stem cells, and outline markers for the identification of stem cells.

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Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; MEF, mouse embryonic fibroblast; NSCs, neural stem cells

Key words: gangliosides, mouse pluripotent stem cells, mouse neural stem cells, mouse neural cells, differentiation, marker

Contents

1. Introduction

Gangliosides belong to a heterogeneous family of lipids known as glycosphingolipids, which are ubiquitously expressed in vertebrate cells, and are particularly abundant in the central nervous system (1). Within mammalian cells, gangliosides are predominantly localized on the plasma membrane (2,3), where they form cell surface microdomains, including caveolae, lipid rafts, and glycolipid-enriched microdomains or cholesterol (2,4,5). Specifically, gangliosides are established to have various functions, including in cell proliferation, differentiation, immune response, adhesion, migration, apoptosis, and cell-cell and cell-substratum interactions (6). Gangliosides are classified by the presence of one or more sialic acid residues linked to different galactose and/or sialic acid residues, and are classified into asialo (o)-, a-, b- and c-series gangliosides, respectively (7).

Stem cells are widely used during research into developmental processes and offer tremendous potential in clinical applications for transplantation and tissue regeneration therapies (8). As they are undifferentiated, stem cells, specifically embryonic

stem cells (ESCs), have a high potential for proliferation (self-renewal) and the capacity to differentiate into various distinct cell types (multipotency or pluripotency) (9). Induced pluripotent stem cells (iPSCs) have been generated from mouse fibroblasts via retroviral introduction of four defined transcription factors: *POU domain, class 5, transcription factor 1* (*Oct-4*), *SRY (sex determining region Y)-box 2* (*Sox-2*), *c-Myc* and *Kruppel-like factor 4* (*Klf-4*) (10). Induction of pluripotency can be also achieved in mouse spermatogonial stem cells by self-reprogramming process (11,12). Reprogrammed pluripotent stem cells, such as iPSCs and germline-derived pluripotent stem cells, are indistinguishable from ESCs in terms of morphology, self-renewal, expression of ESC markers, and their differentiation ability (10,11,13,14). Neural stem cells (NSCs) are known to be self-renewing, multipotent cells that can differentiate into brain-forming cells, such as neurons and glial cells (astrocytes and oligodendrocytes) (15). NSCs highly express nestin, Musashi RNA-binding protein 1 (Musashi-1), Sox-2 and paired box 6 (Pax-6).

2. Stage-specific embryonic antigens (SSEA)

Carbohydrate-associated molecules are known to be involved in controlling cell surface interactions during development. Specifically, SSEA series were originally identified by defined carbohydrate epitopes associated with the lacto- and globo-series glycolipids, such as SSEA-1, SSEA-3 and SSEA-4 (16,17). These SSEA series were expressed in various tissues, cancer and cancer stem cells (18-23). Notably, SSEA series are present in pluripotent stem cells, such as ESCs and iPSCs. SSEA-1 (also termed CD15 and Lewis x) is present on the surface of murine embryos at the pre-implantation stage, in mouse germ cells and on the surface of teratocarcinoma stem cells (24). SSEA-1 is also produced in the thyroid, oviduct epithelium, endometrium and epididymis, and in certain areas of the brain and kidney tubules in adults (18,25). SSEA-1 production increases upon differentiation in human cells and decreases during differentiation in mice.

SSEA-3 and SSEA-4 are synthesized during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage-stage embryos in human (24,26). They are present in undifferentiated primate ESCs, human embryonic germ cells, teratocarcinoma stem cells and ESCs (27).

3. Biosynthesis of gangliosides

Ganglioside biosynthesis and degradation occurs through several events: i) *De novo* ganglioside biosynthesis in the endoplasmic reticulum and Golgi apparatus, followed by vesicular sorting to the plasma membrane; ii) enzyme-assisted chemical modifications of molecules at the plasma membrane level; iii) internalization of gangliosides via endocytosis and recycling to the plasma membrane; iv) direct glycosylations following sorting from endosomes to the Golgi apparatus; v) degradation at the late endosomal/lysosomal level with formation of fragments of sugars (glucose, galactose, hexosamine, sialic acid) and lipids (ceramide, sphingosine, fatty acid); vi) metabolic recycling of these fragments for biosynthetic purposes (salvage pathways); and vii) further degradation of fragments to waste products (Fig. 1) (28).

Ceramides, a group of higher glycosphingolipids, are glucosylated by a glucosyl-transferase (29-31). An uncharacterized flippase enzyme caused Glc-ceramide to flip to the lumenal side of the cis-Golgi stack, where further glycosylations takes place. The first glycosylation, catalyzed by lactosyl (Lac)-ceramide synthase is galactosylation of Glc-ceramide to Lac-ceramide (Fig. 2) (32-34). Lac-ceramide is sialosylated to produce GM3, GD3 and GT3 molecules via the action of three sialyltransferases (SAT) I, II and III, each recognizing their specific acceptor substrate (35,36). GM3, GD3 and GT3 are the starting points for the 'a-series', 'b-series' and 'c-series' gangliosides, respectively. In each ganglioside series, N-acetyl-galactosaminyltransferase, galactosyl-transferase and SAT IV, in sequence, add an N-acetylgalactosamine, galactose and sialic acid group to the gangliosides, respectively, to produce more complex gangliosides. Further sialosylations can be performed by SAT V. From Lac-ceramide a further group of glycosphingolipids ('O-series') can be produced by the sequential action of N-acetyl-galactosaminyltransferase, galactosyl-transferase and sialyl-transferase IV and V, producing asialo-GM2 (GA2), asialo-GM1 (GA1), and gangliosides GM1b, GD1c and GD1α (28).

4. Expression patterns of gangliosides in mouse pluripotent stem cells

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass of blastocysts (37). Established mESCs express various carbohydrate antigens, including glycolipids. Among those, SSEA-1 is the most well-established specific marker.

In E14 and Oct-4 promoter-EGFP (OG2) mESCs, small amounts of a-series gangliosides, GM3, GM1 and GD1a, were detected by high-performance thin-layer chromatography and immunocytochemistry analysis (38,39). Furthermore, in TC-1 mESCs, only glucosylceramide and lactosylceramide were present (40); however, J1 mESCs contained GM3, GM1, and GD3 (Fig. 3A) (41-43). Furthermore, 9-O acetyl GD3 was detected in 129S6/B6-F1/DsRed.T3 mESCs (44). GM3 and GD3 are known to be involved in cell adhesion and proliferation via mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinases (ERK) 1/2 phosphorylation (Table I) (41,42,45,46). Specifically, small hairpin RNA knock-down of UDP-glucose ceramide glucosyltransferase (*UGCG*) to reduce glucosylceramide synthesis was demonstrated to inhibit activation of the Ras-MAPK pathway and cell proliferation (41).

OG2 mouse embryonic fibroblast (MEF) and mESCs produce GM3, GM1, and GD1a, but GM1 is not found in OG2 MEF-derived iPSCs (Fig. 3B) (39). Analysis of the cell proliferation rate in OG2 mESCs and iPSCs revealed that iPSCs have a lower proliferation than that of mESCs. GM1 is known to affect cell proliferation via the ERK 1/2-MAPK pathway and protects against apoptosis in various cell types (Table I) (39,42,47-49).

5. Ganglioside patterns in mouse neural stem cells (mNSCs)

NSCs, also referred to as multipotent neural progenitor cells, can differentiate to cells of the neural linage, including neurons and glial cells (astrocytes and oligodendrocytes) (50). The

Table I. Function and role of gangliosides in mouse stem cells.

Ganglioside	Function/role
GM3	Cell adhesion, proliferation and neural differentiation, induction of neural precursor cells, facilitates neurite formation, neural maturation, activates ERK1/2 MAPK phosphorylation
GM1	Promotes neural differentiation, regulate neurogenesis and regeneration, protects against apoptosis, cell proliferation, activates ERK1/2 MAPK phosphorylation
GD1a	Induction of early neural differentiation
GD3	Induction of early neural differentiation, brain development, cell adhesion and proliferation, neural maturation, facilitates neurite formation, activates ERK1/2-MAPK phosphorylation, induction of neural precursor cells, neural stem cell markers
GT1b	Necessary for induction neural differentiation, enhances actin-rich dendrite generation, increased in brain synapses
GQ1b	Neurite outgrowth during early neural differentiation, neural differentiation through the ERK1/2-MAPK pathway
SSEA-1	Expression on pre- and post-implantation mouse embryo and teratocarcinoma cells, expression in thyroid tissue, expression in human renal tumors
SSEA-3	Expression in human teratocarcinoma cells, expression in colorectal cancer, significant markers for breast cancer stem cells
SSEA-4	Expression in human teratocarcinoma cells, expression in oral cancer cell, expression in basaloid lung cancer

SSEA, stage-specific embryonic antigen; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

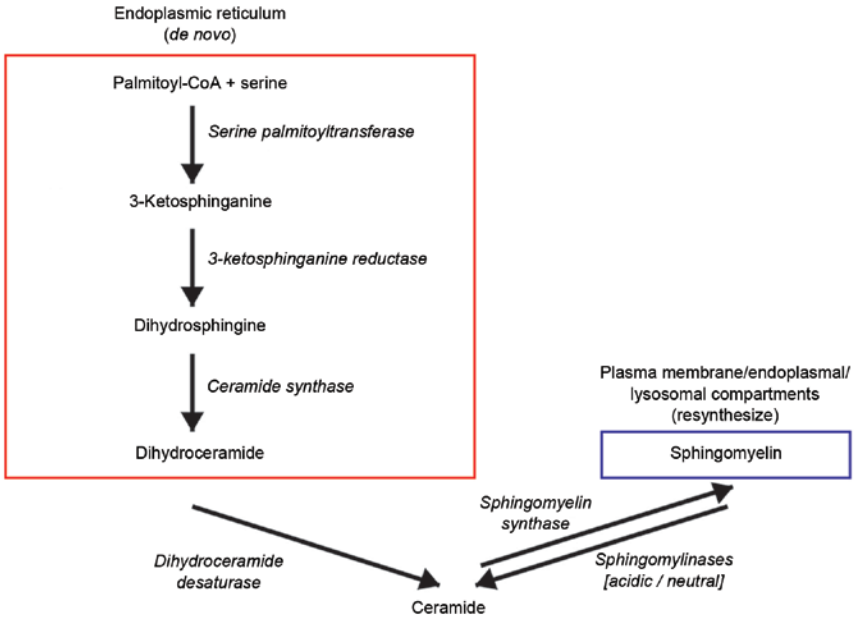

Figure 1. Biochemical pathway of ceramide generation and metabolism. Enzymes are shown in italics. Ceramide is derived from two main pathways operating in different cellular compartments: *De novo* synthesis from serine and palmitoyl CoA; and hydrolysis of membrane-derived sphingomyelin.

NSCs highly express nestin, Musashi-1 and Sox-2; however, these marker molecules are intracellular or nuclear proteins. Several studies demonstrated that there are high levels of b-series gangliosides in NSCs (51-55). GD3 is a b-series disialoganglioside that is frequently detected in vertebrate embryos and immature proliferative cells (56,57). Particularly, GD3 and 9-O acetyl GD3 have been biochemically detected in mNSCs (44,53,58). GD3 is present in the subventricular zone of the lateral ventricle where NSCs are localized (59,60). Furthermore, GD3 expression in mouse neuroepithelial cells is enriched in NSCs, radial glia, embryonic-, postnatal-, and adult-NSCs (Fig. 3C) (53,58,61). Furthermore, NSCs differentiated from mESCs also express GD3 (unpublished data) (Fig. 3A).

The GD3 concentration is known to be high in embryonic brains, which predominantly consist of undifferentiated neural progenitor cells; however, the concentration in the brain rapidly decreases after birth (62). It has been demonstrated

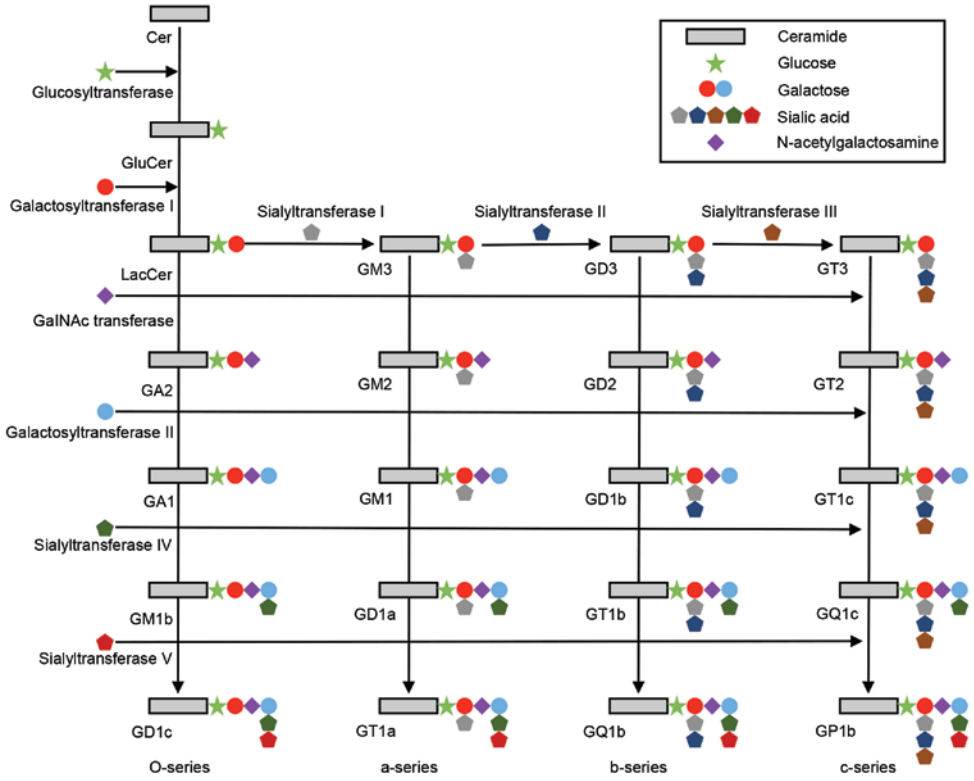

Figure 2. Schematic diagram of the ganglioside biosynthetic pathways. The o-series (GA2, GA1, GM1b and GD1c), the a-series (GM3, GM2, GM1, GD1a and GT1a), the b-series (GD3, GD2, GD1b, GT1b and GQ1b) and the c-series (GT3, GT2, GT1c, GQ1c and GP1c), and the corresponding glucosyltransferase, galactosyltransferases, GalNAc transferase, and sialyltransferases are shown. Cer, ceramide.

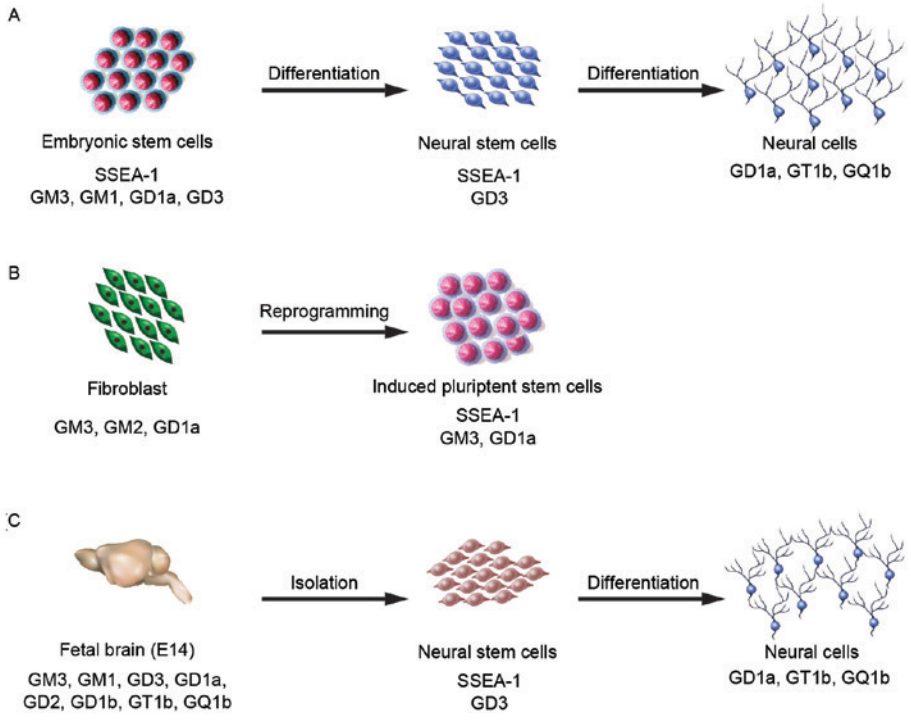

Figure 3. Expression of gangliosides in various mouse stem cells. (A) Patterns of ganglioside synthesis during neural cell differentiation from mouse embryonic stem cells. (B) Changes in ganglioside synthesis patterns from fibroblast to induced pluripotent stem cells during reprogramming. (C) Ganglioside synthesis patterns in fetal brain, isolated neural stem cells from fetal brain and differentiated neural cells. SSEA, stage-specific embryonic antigen; E14, embryonic day 14.

that NSCs derived from GD3-synthase knockout mice could not be maintained in *in vitro* culture (55); thus, it is speculated that GD3 has a major role in the maintenance of NSCs (51,54,63). It has been reported that when the

GD3 level was reduced by an inhibitor of glucosylceramide synthesis, basic fibroblast growth factor-induced proliferation was repressed in primary mNSCs. Additionally, GD3 interacts with epidermal growth factor receptor (EGFR) (55,64). These finding imply that GD3 may induce early neural precursor cell differentiation and neurite formation (Table I) (41,43).

6. Expression patterns of gangliosides in differentiated neural cells

The pattern of ganglioside synthesis changes dramatically during nervous system development (65-67). Thus, gangliosides, including sulfatide (for the myelin sheath in the peripheral and central nerve system), galactosylceramide (for oligodendrocytes) and A2B5 (c-series gangliosides; for neural stem cells, oligodendrocytes, and astrocytes) are considered to be useful as differentiation markers of specific neural lineages (68). It was reported previously that the GD3 level is high in the brain and is involved in embryonic brain development; however, its concentration rapidly decreases during neural development, whereas other gangliosides, including GD1a, GT1b, and GQ1b, increase during aging and neural development (62). In addition, it was demonstrated that correlative changes of ganglioside composition accompany normal development *in vitro* and *in vivo*. Furthermore, b-series gangliosides, including GT1b and GQ1b, are present in mouse neuroepithelial cells (58,69).

Our previous studies demonstrated that GD3, GT1b, and GQ1b are present in cells during retinoic acid-induced neural differentiation of mESCs and embryonic carcinoma cells (Fig. 3A) (42,43). A number of approaches have been reported to determine the role of gangliosides during neural differentiation (70,71). Overproduction of gangliosides can facilitate neurite formation, which is part of the neural maturation process (43). By contrast, knock-down of *Ugcg* was reported to result in a decrease in the neural differentiation rate of mESCs and human dental pulp-derived mesenchymal stem cells (41,72). Therefore, these gangliosides have important regulatory roles in neural differentiation *in vitro* (41-43,73).

GD3 is involved in the early neural differentiation and maturation process (63,74), while GD1a induces early neural differentiation (43). By contrast with GD3, GT1b is necessary for the induction of neural differentiation and drastically enhances actin-rich dendrite generation (42,75). Furthermore, it GT1b syntheses is increased in brain synapses (74). GQ1b promotes neurite outgrowth during early neural differentiation via the ERK 1/2-MAPK pathway (Table I) (43,73,76).

7. Conclusion

Gangliosides are located on the plasma membrane and have roles in various functions of mouse stem cells. As described above, specific gangliosides are detected in mESCs, mouse iPSCs, mNSCs and differentiated neural cells. These gangliosides regulate cell proliferation and differentiation via the MAPK-ERK 1/2 pathway. Furthermore, gangliosides have been demonstrated to be useful marker molecules for detecting or sorting mouse stem cells and differentiated neural cells. Nevertheless, the functional roles of gangliosides during cellular differentiation and proliferation require further investigation. Identification of the gangliosides present in stem cells should be performed to thoroughly characterize marker gangliosides, and contribute to progression in basic stem cell research and clinical applications.

Acknowledgements

This paper was supported by Wonkwang University (Iksan, Korea) in 2015.

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