Chapter 2
Morphogenetic Sphingolipids in Stem Cell Differentiation and Embryo Development

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Abbreviations

Akt	AK strain transforming (Akt kinase)
aPKC	Atypical PKC
C1P	Ceramide-1-phosphate
CECs	Ceramide-enriched compartments
EGF	Endothelial growth factor
ERK	Extracellular regulated kinase
ES cell	Embryonic stem cell
EV	Extracellular vesicle
FB1	Fumonisin B1
FGF-2	Fibroblast growth factor 2
FTY720	Fingolimod
GPCR	G protein-coupled receptor
Grp94	Glucose-regulated protein 94
GSK3	Glycogen synthase kinase 3
GSLs	Glycosphingolipids
HDAC	Histone deacetylase
hESC	Human ES cell
HSP90	Heat shock protein 90
iPSC	Induced pluripotent stem cell
Jak	Janus kinase
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
mESC	Mouse (murine) ES cell

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A. Pébay, R.C.B. Wong (eds.), *Lipidomics of Stem Cells*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-3-319-49343-5_2

NPC	Neural precursor cell
nSMase	Neutral sphingomyelinase
OPC	Oligodendrocyte precursor cells
PAR-4	Prostate apoptosis response 4
PDGF	Platelet-derived growth factor
PDMP	*N*-[2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl]-decanamide
PHB2	Prohibitin 2
PI3K	Phosphatidyl inositol 3 kinase
PIP	Phosphatidyl inositol phosphate
PKC	Protein kinase C
PLC	Phospholipase C
PP2a	Protein phosphatase 2a
S18	*N*-oleoyl serinol
S1P	Sphingosine-1-phosphate
Shh	Sonic hedgehog
SphK	Sphingosine kinase
SPL	S1P lyase
Spns2	Spinster homolog 2
Stat3	Signal transducer and activator of transcription 3
Wnt	Wingless type MMTV

2.1 Ceramide and Its Derivatives

In this section, we will focus on the function of ceramide and derivatives known to regulate stem cell differentiation, namely, sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), and glycosphingolipids (GSLs) (Fig. 2.1). We will not discuss sphingolipid metabolism or the function of sphingolipids in general cell-signaling pathways. There are excellent reviews and the reader is encouraged to attend to these resources [1, 2]. Instead, we will highlight most recent studies showing the function of sphingolipids in cell-signaling pathways critical for regulation of cell polarity and morphogenesis as part of the stem cell differentiation program.

2.1.1 Ceramide and Ceramide-Enriched Compartments

A morphogenetic lipid will induce a specific stem cell differentiation program and regulate embryo development and morphogenesis. We have proposed that ceramide is such a morphogenetic lipid based on the observation that it is critical for the apicobasal patterning of the primitive ectoderm in embryonic stem (ES) cell-derived embryoid bodies and for promoting neural differentiation [2–6]. Compartmentalization into ceramide-enriched compartments, CECs, allows for localized metabolic release

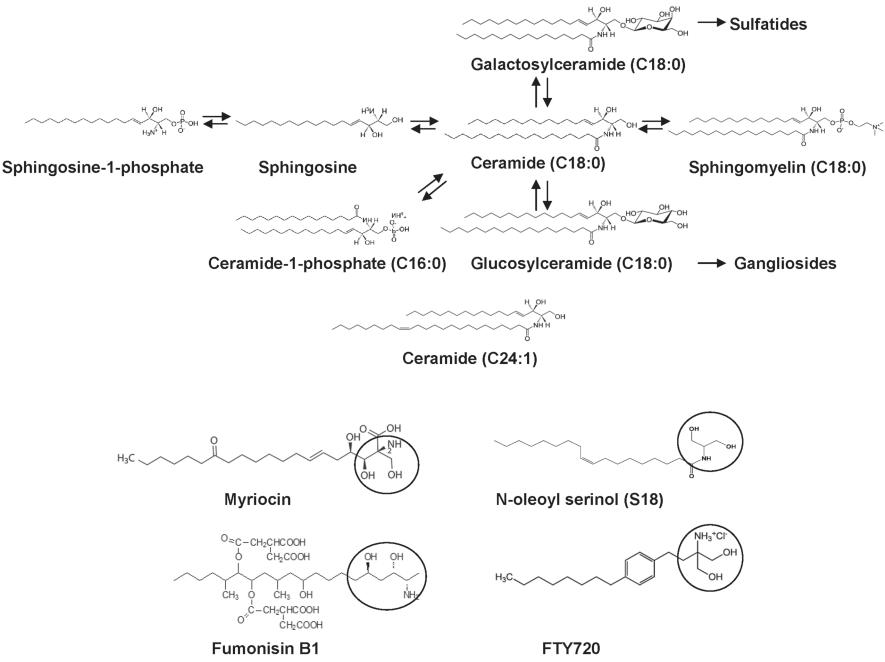

Fig. 2.1. Structure and metabolism of morphogenetic sphingolipids and effectors/analogs. Ceramide is a metabolic hub for the generation of morphogenetic sphingolipids. Myriocin is a serine palmitoyltransferase (SPT) inhibitor. Note the structural difference between C18:0 ceramide (*N*-oleoyl sphingosine) and C24:1 ceramide (*N*-nervonoyl sphingosine). Fumonisin B1 (FB1) is a ceramide synthase inhibitor. FTY720 (fingolimod) is an S1P pro-drug analog. *N*-oleoyl serinol (S18) is a soluble ceramide analog developed in our laboratory. The two β-hydroxy methyl groups (*circled*) of the polar, serine-derived head group are a common structural motif of all ceramide analogs and many other effectors of sphingolipid metabolism

of ceramide derivatives such as ceramide-1-phosphate (C1P, Fig. 2.1) or sphingosine-1-phosphate (S1P, Fig. 2.1), and formation of local sphingolipid-protein complexes that regulate cell polarity. Several years ago, we have termed these hypothetical complexes "sphingolipid-induced protein scaffolds" or SLIPs and proposed their critical function for remodeling of the cytoskeleton and distribution of cell polarity proteins [7]. Recent studies in our and other laboratories support this hypothesis and open the possibility to engineer morphogenesis by changing the composition and compartmentalization of sphingolipids in stem cells.

Our studies and those from other laboratories have demonstrated that sphingolipids including ceramide are organized in lipid microdomains or rafts and CECs [2, 8–18]. In addition, various lipids are distributed in a gradient with cholesterol and sphingomyelin enriched in the cell membrane, while ceramide appears to be enriched in the endosomal compartment [19–21]. Based on these observations, we hypothesize that the lateral anisotropy of sphingolipids leads to raft formation (*X*-axis in Fig. 2.2), which is integrated with a lipid gradient orthogonal to the mem-

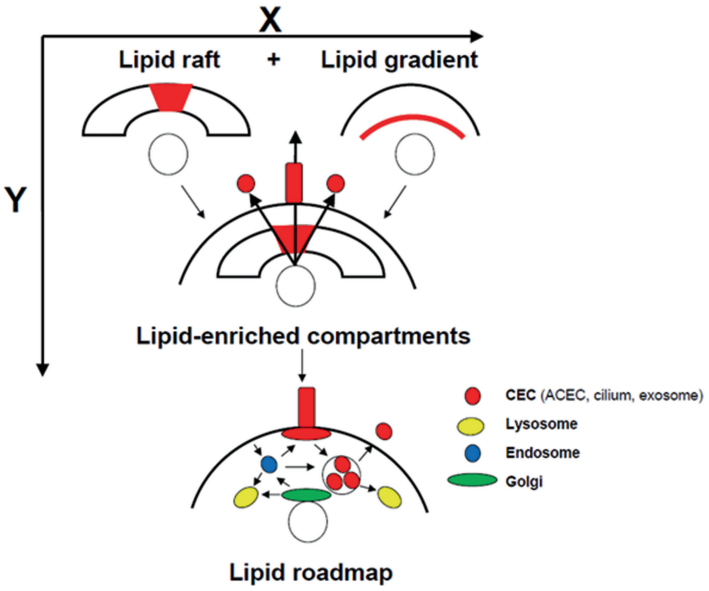

Fig. 2.2. Lipid road map in vesicle trafficking and compartment identity. Integration of lateral membrane anisotropy (lipid rafts or microdomains, here along *X*-axis) with orthogonal lipid gradients (anterograde and retrograde trafficking pathways, here along *Y*-axis) generates a map of vesicles and compartments with distinct lipid composition critical for cell polarity and morphogenesis

brane (*Y*-axis in Fig. 2.2). This integration leads to compartmentalization that regulates intracellular vesicle traffic and polarity similar to a road map directing car traffic (Fig. 2.2, bottom panel). Previous studies noted that sphingolipids are sorted into specific vesicle populations and enriched along distinct trafficking pathways [22–27]. The vesicular identity is even preserved during mitosis when many compartments such as the Golgi apparatus and the nuclear envelope are disintegrated into a myriad of vesicles and yet reassemble in the daughter cells to their original organelles. While only little is known about the sorting mechanisms that direct distinct sphingolipid trafficking pathways toward specific lipid-enriched compartments (including CECs) when exported from the Golgi apparatus/trans-Golgi network or internalized by endocytosis [25, 28–30], one may speculate that they are intimately connected to our model of a lipid road map guiding establishment of cell polarity and ultimately, asymmetric division of progenitor cells and embryo morphogenesis. Our group has shown that two distinct compartments, cilia and exosomes, are enriched with ceramide and directly linked to cell polarity in differentiating stem cells and secretion of growth factors. Formation of these CECs is stimulated by exogenously added ceramide or compromised by inhibitors of enzymes that generate ceramide. Ceramide is enriched at the base and in the membrane of cilia, a cell compartment with sensory and motility functions [4, 11, 31]. It is also enriched in exosomes, lipid vesicles generated in the endosomal compartment and then secreted to transfer cell signaling and growth factors between cells [32].

2.1.1.1 Ceramide and Cilia

Primary cilia are important for stem cell differentiation because they are endowed with growth factor receptors controlling sonic hedgehog, Wnt, FGF, and PDGF cell-signaling pathways [33–53]. Sonic hedgehog binding to its receptor Patched releases the co-receptor Smoothened that is then transported into the cilium and activates the transcription factor Gli, a cilium-controlled process that has been termed "Gli shuttle" [54]. In the neural tube, this mechanism is critical for ventral pattering of the neuroepithelium [33]. In adult neural stem cells and oligodendrocyte precursor cells (OPCs), this mechanism induces the differentiation to neurons and oligodendrocytes, respectively [44, 51, 52]. Factors that regulate ciliogenesis or cilium function are likely to affect and edit these cell-signaling pathways (readers interested in the developmental function of cilia and cilia disorders (ciliopathies) in brain, bone, kidney, and heart are prompted to the following excellent reviews on these topics: [37, 49, 55–61]). While most of research focused on proteins in the regulation of cilia, only very little is known about the function of lipids in ciliogenesis and cilium-induced cell-signaling pathways for stem cell differentiation.

Ceramide is critical for primary cilium formation in mouse and human ES cell-derived neural progenitors [4]. When undifferentiated ES cells were incubated with the ceramide synthase inhibitor Fumonisin B1 (FB1, Fig. 2.1) or the neutral sphingomyelinase (nSMase) inhibitor GW4869, the number and length of primary cilia in neural progenitors were reduced. However, levels of Sox2 and Pax6, two transcription factors expressed in neural progenitors, were not affected. Despite undergoing neural differentiation, progenitors were not able to form rosettes, indicating that loss of ceramide disrupts morphogenesis of the neural tube and ventricular zone during embryonic brain development. Indeed, the *fro/fro* mouse carrying a deletion in *nSMase* shows reduced number and length of ependymal cell motile cilia [31]. Using various inhibitors for ceramide generation including myriocin (Fig. 2.1), FB1 (Fig. 2.1), and GW4869, our group has found that ceramide is not only critical for ciliogenesis, but it is also involved in establishing apicobasal polarity of primitive ectoderm cells and neural progenitors [3, 6].

One of the questions currently investigated in our group is how ceramide regulates the cell-signaling pathways for apicobasal polarity and ciliogenesis. Our working hypothesis is that ceramide enriched in CECs interacts with polarity proteins and the cytoskeleton. Candidate proteins are atypical protein kinase Cζ and ι/λ (aPKC) and glycogen synthase kinase 3β (GSK3), two protein kinases we have shown to bind to ceramide and to regulate acetylation of tubulin in neural cell cilia [3, 10, 31, 62–64]. aPKC as well as GSK3 are also critical for maintaining pluripotency and editing lineage commitment [65–72]. Ceramide binding to these two kinases may very well regulate differentiation of stem cells of various origins. Since ceramide distribution is anisotropic within cellular membranes and even polarized in neural progenitor cells, modulation of aPKC and GSK3 may act through sequestration to CECs and modulation of kinase activity. We have found that the addition of exogenous ceramide, in particular very long chain fatty acid (C24:1) ceramide (Fig. 2.1), increases tubulin acetylation and rescues cilia in neural progenitors with inhibited ceramide biosynthesis [4]. Intriguingly,

acetylated tubulin-labeled processes in ES cell-derived neurons were elongated far beyond 500 μm, indicating that ceramide drives neural differentiation and process formation.

Another ceramide target is protein phosphatase 2A (PP2A). Protein phosphatases were among the first enzymes shown to be activated by ceramide [73–76]. Recent research suggests that ceramide functions to sequester and inactivate the PP2A inhibitor protein I2PP2A in the holoenzyme complex [77]. The significance of the endogenous ceramide–PP2A interaction for stem cell differentiation has not been investigated yet. However, inhibition of PP2A has been reported to sustain self-renewal of stem cells and activation of PP2A by exogenous C2 ceramide has been shown to promote neural differentiation [78, 79]. These observations suggest that activation of PP2A by endogenous ceramide promotes stem cell differentiation toward neural cell fate. PP2A has also been found to increase dephosphorylation of aPKC and GSK3 in *Drosophila* neuroblasts and mammalian cells [79, 80], indicating a synergistic effect with direct binding of these two kinases to ceramide by inactivating (sequestering) aPKC and activating GSK3. In addition to direct effects by binding to PP2A, ceramide can upregulate GSK3 activity by inhibiting the phosphatidylinositol 3 kinase (PI3K)-to-Akt pathway, a major GSK3-inactivating cell-signaling pathway known to sustain self-renewal of stem cells [80, 81]. Taken together, regulation of GSK3 by ceramide involves a variety of cell-signaling networks including aPKC (inactivates GSK3 unless sequestered by ceramide), PI3K/Akt (inactivates GSK3 unless inhibited by ceramide), PP2a (activates GSK3 when activated by ceramide), suggesting that ceramide is a bona fide drug target for enhancing neural differentiation in regenerative medicine.

On a separate note, ceramide appears to be important for both, neuronal and glial differentiation of ES cells, since studies in our laboratory have shown that the combination of exogenously added ceramide (or the ceramide analog *N*-oleoyl serinol, S18, Fig. 2.1) and S1P (or the S1P pro-analog FTY720, Fig. 2.1) directs neural cell fate toward oligodendroglial lineage [82] (for more information on S1P, see following section). In addition, ceramide is critical for primary and motile ciliogenesis in astrocytes and ependymal cells, respectively [4, 31]. In summary, these results suggest that ceramide regulates neural cell fate by a common mechanism that involves ciliogenesis and cell-signaling pathways activated by cilia. Therefore, sonic hedgehog and PDGF are likely candidates to be regulated by ceramide.

2.1.1.2 Ceramide and Exosomes

Exosomes belong to the population of extracellular vesicles (EVs), lipid vesicles that are secreted as intercellular carriers by transporting and transferring proteins, lipids, and RNAs (including microRNAs). In addition to exosomes that are generated in multivesicular endosomes, microvesicles or ectosomes blebbing off the cell membrane constitute another portion of EVs. Ceramide has been shown to be required for the formation and secretion of a particular population of exosomes (ESCORT-independent exosomes) although it is not clear whether there is a specific

function of ceramide-dependent exosomes vs. other EV fractions [32, 83–85]. Our laboratory has shown that exosomes enriched with ceramide, particularly C18:0 ceramide (Fig. 2.1) play important functions in the etiology of Alzheimer's disease [32, 86]. It is not known if stem cells are involved in this process. Cancer stem cells have been shown to secrete exosomes or shed microvesicles to reprogram the host tissue and accommodate metastases [83, 87–90]. This is mainly achieved by the transfer of mRNAs, microRNAs, and enzymes breaking down the extracellular matrix such as matrix metalloproteases.

In principle, stem or progenitor cells could adopt a similar mechanism to either reprogram the tissue in which they differentiate or to receive instructions for differentiation into a particular tissue. In tissue damage and subsequently tissue regeneration, EVs were found to activate stem cells and induce tissue repair [91–95]. In addition, "instructive" exosomes can be custom-made for the use of stem cells in regenerative medicine [96]. In this case, ceramide may primarily be used for boosting instructive exosome formation. It should be noted that the "ciliogenic" C24:1 ceramide (Fig. 2.1) is structurally different from the "exosomogenic" C18:0 ceramide (Fig. 2.1) and that biophysical studies using synthetic lipid vesicles generated with these two ceramide species showed remarkable differences in shaping membranes. While C18:0 ceramide induces spherical shapes, C24:1 ceramide triggers formation of tubules [97, 98]. In astrocyte-derived exosomes, the major ceramides were C18:0 ceramide (ca. 60%) and C24:1 ceramide (ca. 30%) [32]. Therefore, by being enriched in the exosomal membrane, ceramide (especially neuronal process-inducing C24:1 ceramide) may also participate in induction of stem cell differentiation, particularly toward neural lineage as described in the previous section. It should be noted that exosomes are exquisite lipid carriers comparable to liposomes because of their higher surface (membrane)-to-volume ratios, which is dictated by geometry. Currently, the most promising examples for therapeutic use of (stem cell-derived) EVs are cardiovascular wound repair and protection against ischemia-reperfusion injury in heart and kidney [91, 95, 99–102].

2.1.2 Sphingosine-1-Phosphate

Sphingosine-1-phosphate (S1P) is a metabolic derivative of ceramide and another morphogenetic sphingolipid that has a widespread range of biological effects, including regulation of pluripotency and differentiation, survival and proliferation, migration, and homing. S1P regulates the pertinent cell-signaling pathways in various stem cell types, such as pluripotent stem cells, neural stem cells, mesenchymal stem cells, hematopoietic stem cells, endothelial stem cells, and cardiac precursor cells [2, 103–107].

S1P has a short half-life and its tissue levels are maintained by numerous enzymes and factors [103–105]. S1P is mainly generated intracellularly by two enzymes, sphingosine kinase 1 (SphK1) and 2 (SphK2); irreversibly degraded by S1P lyase (SPL); and hydrolyzed by lipid phosphate phosphatases and S1P-specific

phosphatases. It is also exported out of cells by transporter proteins, such as ABC transporters and Spns2 [106, 108–112]. S1P exportation from red blood cells, activated platelets, and endothelial cells comprises most of the extracellular S1P pool, which is usually found at a several-fold higher concentration than that of tissues [112]. SphK1 can also be secreted out and generate S1P outside of cells [112].

Extracellular S1P exerts its function through five cell surface G protein-coupled receptors (GPCRs) S1P1–S1P5 [113] (Fig. 2.3). It stimulates different signal transduction pathways in different cell types depending on the receptors expressed. For example, S1P receptor 1 (S1P1) is coupled exclusively via Gi protein to activate Ras, mitogen-activated protein kinase (MAPK), PI3K/Akt, and phospholipase C pathways [113] (Fig. 2.3). Extracellular S1P has been used to derive or maintain mESCs and hESCs in experimental settings [114–117], demonstrating stimulation of stem cell self-renewal and pluripotency by extracellular S1P. In mESCs, the main pathway allowing maintenance of pluripotency appears to be through the activation of the JAK/STAT3 pathway [117–119]. This notion is supported by studies showing that silencing of the S1P-degrading enzyme, SPL, leads to an increased S1P level concomitant with increased proliferation, and elevated expression of pluripotency markers Ssea1 and Oct-4 in mESCs [120]. The S1P2/Stat3 signaling has been identified to be the major pathway in SPL knockdown-mediated pluripotency. Besides pluripotency maintenance, extracellular S1P plays other crucial roles in stem cells, including proliferation, migration, and homing of various types of progenitor cells (see reviews by [109, 121–124]), and it is critical for vascular development ([109, 125, 126] and reviews by [123, 124]). Extracelluar S1P signaling is important for tumorigenesis and holds great potential as target for disease treatment [105]. S1P promotes cancer stem cell generation and expansion, which contributes greatly to drug resistance, metastasis, and relapse in multiple cancer types [127, 128].

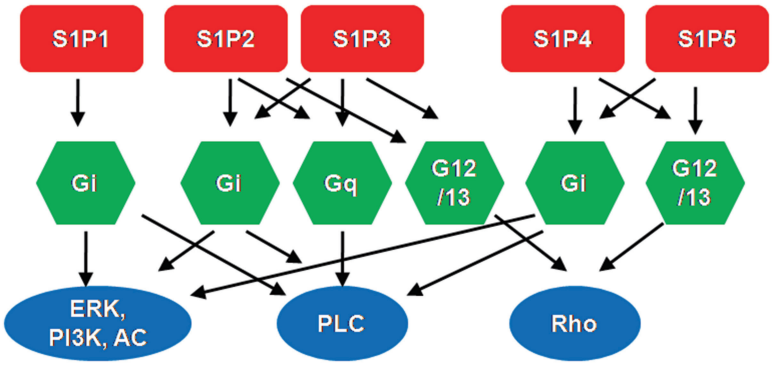

Fig. 2.3. Signaling pathways regulated by extracellular S1P. Extracellular S1P is a ligand for five specific G protein-coupled receptors $S1P_1$–$S1P_5$. Each S1P receptor is coupled to different G proteins; G_i,G_q, $G_{12–13}$, which regulates stem cell pluripotency, self-renewal, and differentiation through various kinases such as ERK (extracellular signal-regulated kinases), PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase), AC (adenylyl cyclase), PLC (phospholipase C), and Rho GTPase

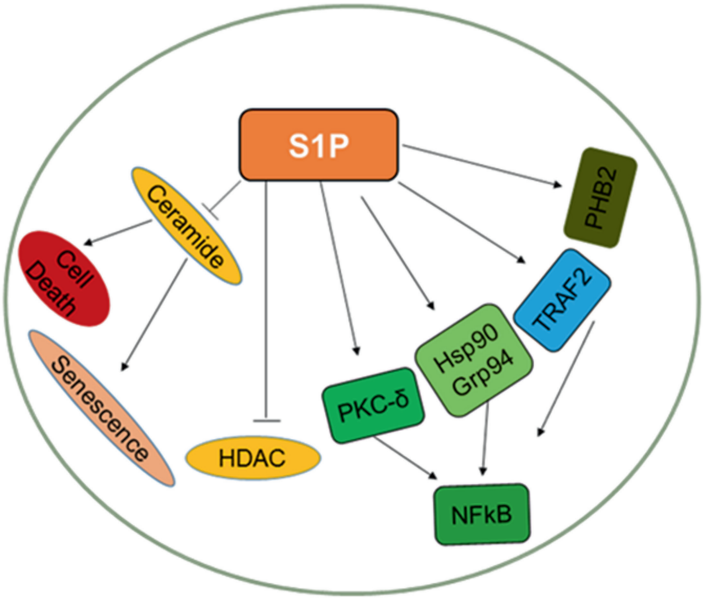

Fig. 2.4. Signaling pathways regulated by intracellular S1P. Intracellular S1P regulates stem cell fate through intracellular targets ceramide, HDAC (histone deacetylases, nuclear), Hsp90 (heat shock protein 90, cytosolic), Grp94 (glucose-regulated protein 94, ER), PHB2 (prohibitin 2, mitochondria), PKCδ (protein kinase C δ, cytosolic), and potentially TRAF2 (TNF receptor associated factor 2, cytosolic)

On the other hand, S1P-primed human mesenchymal stem cells enhance therapeutic potential for pulmonary artery hypertension [129].

Intracellular S1P carries out its function in a receptor-independent manner [104], by either mediating calcium release from the endoplasmic reticulum, or by interacting with its intracellular targets, such as PKCδ, histone deacetylases (HDACs), prohibitin 2 (PHB2), Grp94, and Hsp90α [130, 131] (Fig. 2.4). The intracellular S1P target, PKCδ, is essential for stem cell maintenance and differentiation. Activation of PKCδ mediates cardiac differentiation from ESCs and hematopoietic stem cells [132, 133]. Further, PKCδ activity is required for Jagged-1 induced osteoblast differentiation in hESCs together with canonical Notch signaling [134]. With respect to the function of PKCδ in stem cell pluripotency, it has been found that treatment with PKCδ inhibitors, GF 109203X and rottlerin, prevents early differentiation of mESCs undergoing hypoxia by increasing levels of leukemia inhibitory factor (LIF) receptor and phosphorylated Stat3 [135]. These studies were validated in human pluripotent stem cells by a kinase inhibitor library screening, which identifies PKC inhibitors capable of enhancing pluripotency [136]. Another intracellular target of S1P is histone deacetylase (HDAC). It is known that epigenetic landscapes determine stem cell fate (see reviews [137, 138]). HDACs form the core catalytic component of co-repressor complexes that epigenetically regulate gene expression. Deletion of HDAC1 and

HDAC2 in ES cells caused cell death specifically in undifferentiated cells, concomitant with drastic reduction of pluripotency factors Oct-4, Nanog, Esrrb, and Rex1, indicating that HDAC1 and HDAC2 are essential for pluripotency and renewal of embryonic stem cells [139]. During stem cell differentiation, HDAC inhibition increases expression of neuroectodermal markers and enhances the neuroectodermal specification once neural differentiation is initiated, thereby leading to more neural progenitor cell generation.

In addition to HDACs, other intracellular target proteins of S1P have been identified. S1P activates Prohibitin 2 (PHB2). PHB2 is a pleiotropic factor mainly localized in mitochondria. PHB2 is highly expressed in pluripotent mESCs and decreased during differentiation. Knockdown of PHB2 leads to significant apoptosis, whereas its overexpression results in enhanced proliferation. These results suggest that PHB2 is a crucial regulatory factor for homeostasis and differentiation in mES cells [140]. Similarly, in flat worms (planarians), silencing of PHB2 greatly reduced the number of proliferating neoblasts, which severely impairs tissue regeneration [141]. The Hsp90 family members Hsp90α and Grp94 are newly identified intracellular S1P target proteins [131]. S1P specifically interacts with the N-terminal domain of heat shock proteins during ER stress [131]. Both Hsp90 and Grp94 are essential regulators of stem cell fate. Pharmacological inhibition and genetic knockdown of Hsp90 leads to pluripotency loss in mESCs, which is rescued by Hsp90 re-expression [118]. Hsp90 associates with Oct-4 and Nanog and protects them from degradation by the ubiquitin proteasome system [118]. Hsp90 inhibition predominantly leads to mesoderm differentiation. Because of these effects, Hsp90 inhibitors have been used to specifically eliminate cancer stem cells in a wide range of cancer types [142, 143]. On the other hand, Grp94 deletion leads to defects in mesoderm formation in mice as well as mESCs [144]. Liver-specific deletion of GRP94 leads to hyperproliferation of progenitor cells and acceleration of tumor development in a PTEN-dependent manner, including both hepatocellular carcinoma and cholangiocarcinoma, suggestive of progenitor cell origin [145]. In summary, both intra- and extracellular S1P play profound roles in stem cell biology, which in turn contributes significantly to normal development, morphogenesis, and disease initiation and treatment.

2.1.3 Ceramide-1-Phosphate

Ceramide-1-phosphate (C1P) is synthesized from ceramide by ceramide kinase (Fig. 2.1). It has been shown to induce migration of mesenchymal and hematopoietic stem cells although studies on embryonic stem cells or embryo development are not yet available [146–148]. Its potential as sphingolipid being important for stem cell differentiation (and potentially, morphogenesis) may emerge from its ability to activate phospholipase A_2, an enzyme generating lysophosphatidic acid (LPA) and arachidonic acid, the precursor of eicosanoids [149–151]. Both LPA and eicosanoids involved in stem cell differentiation will be discussed in other chapters of this book.

2.1.4 Glycosphingolipids

Glycosphingolipids (GSLs) are a major class of ceramide derivatives important for differentiation of stem and progenitor cells. Their biosynthesis starts with glycosylation of the C1 hydroxyl group of ceramide using activated glucose or galactose, which can then be followed by the addition of other sugar residues that are either neutral (neutral GSLs) or modified by acidic groups (sulfatides and complex GSLs) (Fig. 2.1). Galactosylceramide is the main (neutral) GSL in brain and comprises about 23% of the total mass of myelin lipids [152]. Galactosylceramide is also known as O1 epitope, a marker for immature oligodendrocytes and the metabolic precursor for galactosulfatide (O4 epitope), a marker for OPCs [153–155]. Determination or isolation of OPCs and oligodendrocytes is achieved by detecting and separating cells with O4(+)/O1(−) and O4(+)/O1(+) epitopes, respectively. Interestingly, the O4 (but not O1) antibody can block terminal differentiation of oligodendrocytes, indicating a functional role of galactosulfatide in differentiation [156, 157].

Galactosulfatide has been suggested to mediate axon-glial contact at the node of Ranvier, a site were the myelin sheath attaches to the axon and leaves a gap for saltatory conduction of the electrical current along the nerve fiber [158–160]. The role of galactosulfatide in OPC differentiation is unclear, while the function of its precursor galactosylceramide is better characterized. It has been reported that galactosylceramides form lipid microdomains or rafts with two other lipids, cholesterol and sphingomyelin in the membrane of the endoplasmic reticulum of OPCs and other cells [161–163]. These lipid rafts interact with sigma receptors important for OPC differentiation. It is not known if galactosulfatide forms lipid rafts as well [161].

In contrast to galactosulfatide, the function of other GSLs, particularly globosides and gangliosides in the regulation of growth factor receptors by lipid rafts is well investigated. Globosides and gangliosides are synthesized from glucosylceramide by first adding galactose (forms lactosylceramide) and then other sugar residues with modification, particularly N-acetyl residues (Fig. 2.1). A rather simple ganglioside termed GD3 has been found to be highly enriched in neural stem cells and to activate EGF receptors in lipid rafts of the plasma membrane [164–169]. Another more complex ganglioside, GM1, has been shown to activate calcium influx into nuclei, which is likely to involve lipid rafts and interaction of Na/Ca exchangers with GM1 in the nuclear membrane [170–174]. While GD3 promotes self-renewal of neural stem and progenitor cells, GM1-induced calcium influx triggers neural differentiation and sustains function of mature neurons. Consistent with consecutive stages of neural differentiation, ganglioside biosynthesis switches from simpler to more complex gangliosides at gestational day E14.5 (mouse), a time point when neural progenitor cells start to divide asymmetrically and give rise to one self-renewing daughter stem cell and one intermediate progenitor eventually undergoing terminal differentiation [175, 176]. We have found that at this time point in brain development, ceramide is also upregulated, suggesting integration of sphingolipid metabolism with neural differentiation [177].

Consistent with the importance of sphingolipid metabolism for neural differentiation, knockout mice for enzymes in ceramide or ganglioside biosynthesis show defects in brain development or function [16, 178–183]. Due to metabolic and functional redundancy (several enzymes can generate the same lipid or different lipids have similar functions), the phenotypes of these knockout mice are not always as severe as predicted by functions determined in vitro. In fact, it appears that the severity of ceramide synthase and glycosyltransferase knockout mice in ceramide and ganglioside biosynthesis is more visible during adult neural differentiation and function than in embryo development. The knockout mice described for deletion of ceramide synthase 1 and 2, glucosylceramide synthase, and alkaline ceramidase 3 are deficient in cerebellar function, particularly due to Purkinje neuron defects or loss [184–189]. The phenotype of the ceramidase synthase 1-deficienct mouse resembles that of the alkaline ceramidase 3 knockout, suggesting that ceramide imbalance is detrimental for adult neural differentiation and function [184, 188]. However, in the ceramide synthase knockout mice, deficiency of a particular ceramide species is accompanied by accumulation of the immediate metabolic ceramide precursors, the long chain bases sphingosine and dihydrosphingosine [188, 190]. Most recently, it was shown that expressing ceramide synthase 2 in the background of ceramide synthase 1 knockout leads to normalization of the long chain bases sphingosine and dihydrosphingosine, while total ceramide levels were not affected [190]. This observation suggests that the phenotype of ceramide synthase knockouts is rather caused by accumulation of long chain bases than lack of ceramide. Interestingly, neurotoxicity of long chain bases has already been described decades ago when the fungus toxin fumonisin B1 (FB1) was found in *Fusarium*-contaminated corn or food for kettle and horses [191–194]. FB1 is a specific inhibitor of ceramide synthases, which leads to reduction of total ceramide and increase of long chain base concentration. In rural areas of South America, eating tortillas contaminated with *Fusarium* leads to a high rate of birth defects, particularly neural tube closure defects and *spina bifida* [195]. This phenotype resembles genetic deficiencies in the Shh pathway, which we already discussed to be activated by primary cilia, and potentially ceramide as regulator for ciliogenesis [196–198]. Currently, it is not known why increased levels of long chain bases or decreased ceramide levels affect neural development, but the phenotypes of the respective knockout mice and effects of inhibitors in ceramide biosynthesis clearly indicate that regulation of sphingolipid metabolism is critical for neural differentiation and function.

2.1.5 Sphingolipids in Stem Cell Therapy and Regenerative Medicine

The plethora of developmental processes regulated by sphingolipids suggests that they are useful in regenerative medicine, particularly for the controlled differentiation of stem cells. Currently, there are three potential avenues tested or hypothetically useful for the application of sphingolipids in stem cell differentiation and

regenerative medicine: (1) direct administration of sphingolipids or analogs; (2) generation and administration of sphingolipid-enriched exosomes; and (3) administration of effectors for enzymes in sphingolipid metabolism. Sphingolipids/analogs, exosomes, and enzyme effectors can be added to stem cells in vitro prior to grafting or in vivo, directly into the recipient organism prior to, after, or without stem cell transplantation. Research in our laboratory has focused on in vitro treatment of pluripotent stem cells with ceramide and S1P analogs prior to transplantation into brain. In many ES cell-derived progenitor cell preparations, residual pluripotent stem cells pose the risk of teratoma or other tumor formation after transplantation [199]. We discovered that escaping from apoptosis is one of the reasons why residual pluripotent or progenitor cells (termed "Zombie cells") continue to proliferate [200]. Once apoptosis is reactivated by incubation of progenitors with ceramide analogs, particularly *N*-oleoyl serinol or S18 (Fig. 2.1), the risk of teratoma formation is dramatically reduced. In follow-up studies, we observed that incubation of S18-treated stem cells with the S1P pro-analog FTY720 (Fig. 2.1) directs neural differentiation toward oligodendroglial lineage [5, 82]. Our results suggest that the expression level of prostate apoptosis response-4 (PAR-4), a sensitizer toward ceramide-induced apoptosis, is critical for this specificity. In contrast to residual pluripotent cells with higher PAR-4 expression levels, neural progenitors express only little of PAR-4, while they express the S1P and FTY720 receptor S1P1 (Edg-1), which promotes oligodendrocyte differentiation [5].

The use of FTY720 in improving oligodendrocyte differentiation or function has been hypothesized to be in part responsible for the beneficial effect of fingolimod, the medical preparation of FTY720, in treating multiple sclerosis (MS). The main effect of FTY720 is induction of endocytosis and proteolytic degradation of S1P1 in peripheral T-cells that account for the autoimmune response destroying myelin in MS patients [201]. However, recent research suggests that FTY720 has additional effects on the central nervous system due to its ability to penetrate the blood–brain barrier. For one, it has been found to downregulate S1P1 in reactive astrocytes, which suppresses neuroinflammation aggravating MS. [202, 203] Secondly, it has been shown to protect NPCs and OPCs due to its activating effect on S1P1 [5, 204, 205]. Most likely, the outcome of FTY720 depends on the effective dose and duration of incubation. At low nanomolar concentration and short incubation time, it will activate S1P1 and protect and promote differentiation of OPCs, while at higher concentration and longer incubation time, it will induce S1P1 receptor degradation and prevent neuroinflammation. More recently, several additional molecular targets of FTY720 have been identified, including ceramide synthase (inhibited by FTY720) and PP2A (activated by FTY720), turning this drug into a promising "magic bullet" for treatment of several CNS diseases and cancer [206–210].

While direct administration of sphingolipid analogs to stem cells or in vivo is one potential application, the use of exosomes is another one that rapidly gains interest in regenerative medicine. So far, two avenues have been tested: (1) administration of exosomes to stem cells prior to grafting, and (2) direct injection of exosomes into the blood stream. Exosomes can be stem cell-derived ("stem cell therapy without stem cells") or they can be custom-made and produced by any other appropriate cell

type [91–95, 211]. Of the >100 papers currently published on the topic of exosomes in regenerative medicine, the majority focuses on designing exosomes carrying specific microRNAs to reprogram stem cells in vitro and in vivo. Only little is known on the use of sphingolipids in exosome therapy.

Last not least, effectors of sphingolipid metabolism can be directly used in stem cells to "metabolically reprogram" their identity, enhance safety, or boost differentiation toward a particular lineage. While promising in theory, this approach has not yet found significant practical application. The reason maybe twofold: (1) most known effectors of sphingolipid metabolism are enzyme inhibitors that prevent biosynthesis of sphingolipids useful for stem cell differentiation such as ceramide, S1P, and gangliosides; and (2) once biosynthesis of a particular sphingolipid is inhibited, a wealth of important metabolic derivatives of this sphinoglipid are also depleted. Enzyme inhibitors have not found widespread use to manipulate sphingolipid metabolism in stem cells. However, there are anecdotal reports that may change this. D-PDMP, a specific inhibitor of glucosyltransferase, the enzyme that converts ceramide to glucosylceramide, has been applied to neural progenitor cells, but without significant effect on neural differentiation [212]. The non-inhibitor stereoisomer L-PDMP, however, was shown to stimulate neural progenitor proliferation in vitro and in vivo [213–215]. It has been suggested that in contrast to D-PDMP, L-PDMP stimulates glucosylceramide and ganglioside biosynthesis, but it is not known if this compound can be used to enhance stem cells for therapy. In principle, a combination of enzyme inhibitors and sphingolipid analogs can be used to tailor the sphingolipid composition in stem cells and control differentiation. Future studies are needed to determine if this approach is beneficial in stem cell therapy and regenerative medicine.

2.2 Other Lipids

Apart from sphingolipids, many other lipids are known to regulate stem cell differentiation and embryo morphogenesis. These lipids can be post-translational modifications of cell-signaling proteins (e.g., palmitoylation), receptor ligands (e.g., eicosanoids), or cell-signaling lipids to activate or inhibit cell-signaling pathways (e.g., phosphatidylinositol phosphates or PIPs) that sustain self-renewal or promote differentiation of stem and progenitor cells [2]. These lipids often form lipid microdomains or rafts together with sphingolipids due to membrane anisotropy. Therefore, they can cooperate with sphingolipids in editing cell-signaling pathways for stem cell differentiation and morphogenesis. Among lipid modifications of cell-signaling proteins, palmitoylation and cholesterylation of Shh is probably the most prominent example [216, 217]. Cholesterol derivatives such as steroids, as well as eicosanoids and retinoic acid almost exclusively act through receptors. PIPs activate protein kinases in the stem cell survival pathway and promote differentiation toward specific lineages [218, 219]. Similar to ceramide, PIPs are not only cell signaling but also polarity lipids in that their asymmetric distribution recruits and locally activates

kinases in the regulation of cell polarity and migration. The integration of cell differentiation and polarity is vital for germ layer formation and embryo morphogenesis. Similar to sphingolipids, generation and localization of other lipids, including cholesterol, eicosanoids, and PIPs is controlled by enzymes in the respective lipid metabolism, which allows for metabolic integration of stem cell metabolism and differentiation.

2.3 Concluding Remarks

The effect of sphingolipids on stem cell differentiation is far more diverse than one could do justice in just one single review or book chapter. However, in order to define an overarching function for lipids in differentiation and development one should let go of discussing these effects for individual lipid classes. We believe that after finishing this chapter, one conclusion can be safely drawn: unlike many proteins with narrowly defined functions, lipids often have overlapping functions and can complement or substitute for each other, regardless of being sphingolipids or other lipid classes. So, what is the "bigger picture" in the role of lipids for stem cell differentiation and development? Why do different lipids have similar effects and can complement or even substitute for each other? And how is this overarching function useful in regenerative medicine to improve stem cells?

In contrast to most proteins, the biosynthesis of which is initiated outside of the membrane, lipids are intrinsic constituents of cellular membranes. Many lipids do not have to be made and then inserted, they are of membrane origin. To change lipid composition, membranes are fused or membrane-resident lipids converted by enzymes. Therefore, lipids are the root cause for determining membrane fluidity and anisotropy, even if regulated by localized enzyme activation or spatially directed vesicle transport. This membrane anisotropy can show itself by localized clustering as in lipid rafts or even asymmetry as in apicobasal polarity or localized membrane protrusions such as cilia and neuronal processes. Membrane anisotropy may rely on lipids in self-organized domains or rafts, involve cytoskeletal and motor proteins that move rafts and vesicles, or endow proteins with lipid moieties to attach to rafts and form spatial gradients and locally defined cell-signaling platforms. Based on these few considerations, one may conclude that the main contributions of lipids to stem cell differentiation and embryo morphogenesis is to endow stem and progenitor cells with polarity, a spatial cue that gives cells orientation in a bigger complex made of constantly morphing layers and tissues during development. Therefore, the term "morphogenetic lipids" is about the function of lipids in the integration of stem cell differentiation and embryo morphogenesis.

How can this function of lipids be utilized in designing differentiation protocols that improve stem cell therapy for regenerative medicine? The linchpin of lipid-regulated stem cell differentiation and its integration with morphogenesis is the association of membrane anisotropy with regulation of the cytoskeleton and cell polarity. Membrane anisotropy is initiated by the formation of lipid microdomains

or rafts. Lipid rafts can be self-organized by the biophysical properties of lipids; this has been shown by a plethora of experiments using synthetic vesicles made of pure lipid compositions [15, 98, 220–224]. However, the way rafts morph, move, and interact with other membrane components needs the participation of proteins in a mutually regulating process.

Interestingly, the consequence of this rather inclusive view is that "next generation design" of stem cells in regenerative medicine will rely on reagent cocktails that include effectors for lipid metabolism as well as the associated protein signaling. In a somewhat surprising way, this has already been done from the very beginning of stem cell research. Colchicine, a microtubule-destabilizing drug, has been used to prevent neural differentiation of P19 teratocarcinoma and other types of undifferentiated stem cells [225–227]. Once commitment to neural progenitors is initiated by incubation with retinoic acid, cells become resistant due to acetylation- and detyrosination-induced stabilization of microtubules and incorporation of neurofilaments and microtubule-associated proteins [225, 227, 228]. Retinoic acid induces a several-fold increase in the levels of ceramide in teratocarcinoma cells, which has previously been considered a pro-apoptotic signal [229]. However, we have discovered that very long chain C24:1 ceramide is upregulated during neural differentiation of human ES and iPS cells and promotes acetylation of microtubules due to downregulation or inhibition of HDAC6 [4] (see also above for discussion of ceramide in ciliogenesis). Hence, ceramide may act through a dual effect on promoting neuronal differentiation and concurrent stabilization of microtubules by inhibiting deacetylation. Likewise, another ceramide target recently discovered, GSK3, may promote differentiation through the canonical Wnt/β-catenin cell-signaling pathway as well as increased outgrowth of neuronal processes through the non-canonical pathway and tubulin acetylation through inhibition of HDAC6, respectively.

The GPCR-to-PI3K/Akt-to-GSK3 cell-signaling pathway is one of the major signaling hubs interfacing induction of stem cell differentiation by growth factors with sphingolipid metabolism. Recent studies from our and other laboratories show that this pathway is a node for integrating sphingolipid (S1P and ceramide) and LPA with PIP signaling since S1P and LPA act on GPCRs and inactivate GSK3 through activation of Akt by PIP3 (Fig. 2.5). S1P or LPA counteract ceramide-mediated inhibition of Akt by GPCR-mediated activation of PI3K/Akt. Based on these observations, we conclude that Akt and GSK3-regulated differentiation of stem cells and embryo morphogenesis is balanced by S1P (leads to activation of Akt, inactivation of GSK3, and self-renewal) and ceramide (leads to inactivation of Akt, activation of GSK3, and differentiation). Pharmacological inhibition of Akt with LY294002 and GSK3 with bio/indirubin monoxime has been shown to promote differentiation and pluripotency, respectively [69, 81]. It should be noted, however, that the effect of Akt and GSK3 inhibitors is differential and has opposite effects depending on the duration of incubation or developmental stage. Long-term inhibitor incubation or inhibition of Akt and GSK3 at more committed progenitor stages will prevent differentiation and self-renewal, respectively [65, 230–232].

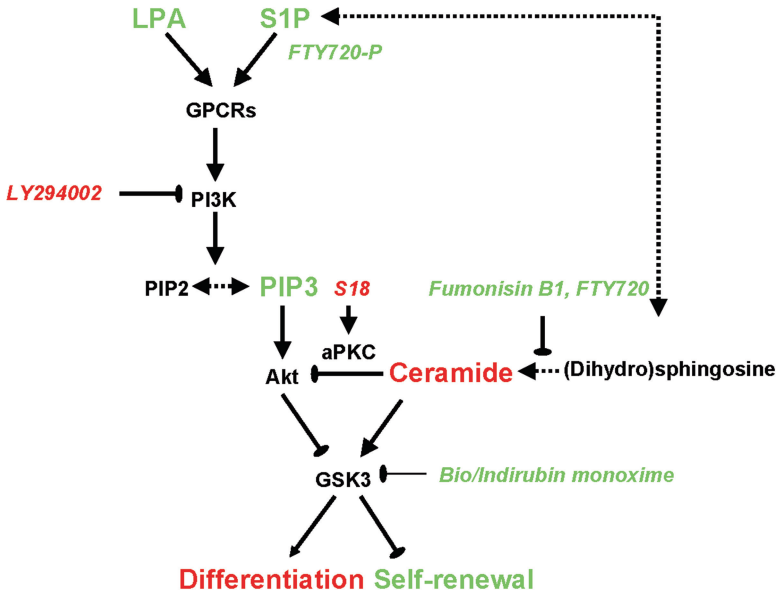

Fig. 2.5. Lipid-regulated GPCR-to-PI3K/Akt-to-GSK3 cell-signaling pathways modulate cell fate decisions in stem cells and morphogenesis. The balance between ceramide and S1P regulates cell fate decision between self-renewal and differentiation in stem cells through different signaling nodes in the GPCR-to-PI3K/Akt-to-GSK3 cells-signaling pathway

The outcome of the GPCR-to-PI3K/Akt-to-GSK3 cell-signaling node is mostly modulated by two growth factors, LIF and fibroblast growth factor-2 (FGF-2), and the pertinent downstream activation of additional cell-signaling pathways, particularly the JAK/STAT3 (via LIF) and ERK (via FGF-2) pathways [66, 81, 233]. Because mouse and human stem cells differ in their response to these growth factors, it is difficult to predict and requires empirical testing to determine which combination of growth factor and modulator of lipid cell-signaling pathways will direct stem cell fate to a desired cell type.

Our research has shown that ceramide may bind and activate GSK3 and in turn, promote acetylation of microtubules and neuronal process formation [4, 31]. On the other hand, we have also found that during differentiation of neural stem cells to OPCs, S1P and ceramide or its analog *N*-oleoyl serinol (S18, Fig. 2.1) may act synergistically once progenitors are committed to glial cell fate [2, 5, 82] (Fig. 2.5). Since S1P can be metabolically derived from ceramide (and vice versa) (Figs. 2.1 and 2.5), sphingolipid metabolism will play an important role in the regulation of stem cell differentiation. The metabolic balance between S1P and ceramide, once predominantly linked to the decision between cell survival and death, has gained a far more subtle and novel function in stem cell differentiation and embryo morphogenesis. Therefore, sphingolipids, particularly S1P and ceramide are morphogenetic lipids and potential drug targets for regenerative medicine.

Acknowledgments This study was supported by grants NIH R01AG034389, R01NS095215, and NSF1121579 to E.B. and American Lung Association RG-351596 to G.W. We are also grateful to institutional support by the Department of Neuroscience and Regenerative Medicine (chair Dr. Lin Mei), Medical College of Georgia at Augusta University.

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http://www.springer.com/978-3-319-49342-8

Lipidomics of Stem Cells

Pébay, A.; Wong, R.C. (Eds.)

2017, XIII, 212 p. 24 illus., 22 illus. in color., Hardcover
ISBN: 978-3-319-49342-8
A product of Humana Press