More than two decades ago S1P was first suggested to be an intracellular second messenger for Ca^{2+} mobilization in the field of signal transduction [1]. Around the same time Spiegel and colleagues [2] found that S1P induced a transient Ca^{2+} release from the intracellular Ca^{2+} store and stimulated DNA synthesis in Swiss 3T3 fibroblasts. They previously observed that sphingosine, which had been suggested to be an endogenous inhibitor of protein kinase C [3], unexpectedly stimulated DNA synthesis, depending on the concentration employed [4].
They showed that sphingosine was phosphorylated by sphingosine kinase (SphK) to become S1P before exerting mitogenic effect. Indeed, platelet-derived growth factor (PDGF) and serum mitogens induced a transient activation of SphK in cultured fibroblasts, leading to a proposal that S1P generated intracellularly acted as a second messenger for mitogenesis [5].

Igarashi and colleagues reported, on the other hand, that S1P potently inhibited B16 melanoma cell migration and invasion of Matrigel [6]. They suggested that S1P acted via a putative cell surface receptor rather than as a second messenger, based upon their observations including effectiveness of immobilized S1P and detection of saturable high affinity cell surface binding sites for S1P [7].

These pioneering works disclosed two distinct, major aspects of S1P actions, i.e. stimulation of cell proliferation and inhibition of cell migration, which are now proven to exert stimulatory and inhibitory effects, respectively, in tumor progression via different subtypes of the G protein-coupled S1P receptors.

It is now well established that the S1P signaling system plays crucial roles in mammalian development and physiology, maintaining homeostasis of such diverse systems as cardiovascular, immune, respiratory, endocrine, reproductive and nervous systems, and liver, kidney, bone and so on; it is also implicated in human diseases including cancer and atherosclerosis, the two leading causes of death in developed countries, among many others [8-19]. The S1P signaling system consists of S1P synthesizing and degrading enzymes, transmembrane S1P transporters and S1P carrier proteins in the plasma, in addition to five members of the G protein-coupled S1P receptors.

In this review, we will briefly overview basic architectures of the S1P signaling through three ubiquitously expressed S1P receptor (S1PR) subtypes, and then focus on S1PRs in tumor cells, S1PRs in host cells in the context of tumor angiogenesis. We will also overview molecular mechanisms for overexpression of sphingosine kinase 1 (SphK1) in tumor cells and its roles in tumor progression, which include sustained activation of Akt, stabilization of HIF1α and S1P production.

Identification of the G protein-coupled receptor (GPCR) family for S1P and LPA

The lysosphingolipid S1P shows structural similarity to lysophosphatidic acid (LPA), which is a classical plasma lipid mediator with pleiotropic activities [20-26]. Moolenaar and colleagues first demonstrated that LPA stimulated DNA synthesis and phosphoinositide hydrolysis with cellular Ca2+ mobilization in pertussis toxin-sensitive and -insensitive manners, respectively, in fibroblasts [23]. Their observations and other studies also indicated that a transient intracellular Ca2+ mobilization at the G0/G1 interface was neither required nor sufficient as a mitogenic signal in fibroblasts and other cell types [23, 27-31]. In N1E-115 neuronal cells LPA induced neurite retraction in a C3 toxin-sensitive, Rho-dependent manner [32]. Exogenously added S1P, but not its microinjection, was also shown to induce neurite retraction and Ca2+ mobilization, with the latter showing homologous desensitization but not cross-desensitization to LPA [33]. In guinea pig atrial myocytes S1P but not LPA activated Ik(Ach) in a pertussis toxin-sensitive manner [25]. These observations strongly suggested the existence of distinct, yet closely related, GPCRs for S1P and LPA.

In an attempt to explore a novel signaling system in the vasculature our group cloned a putative GPCR from rat aortic cDNA library [34]. This clone, designated as AGR16 (=EDG5/H218/S1PR2), was abundantly expressed in vascular smooth muscle cells but showed no similarity to any known GPCR except for EDG1 (=S1PR1), which was reported by Hla and Maciag [35] as an mRNA upregulated in differentiating human umbilical vein endothelial cells (HUVECs) in response to a phorbol ester. Chun and colleagues identified vzg-1 (=EDG2/LPA1), which has a substantial homology with EDG1 and EDG5, as a GPCR specific for LPA [36]. After this discovery, EDG1, EDG5, and another closely related one (S1PR3/EDG3), were identified as receptors specific for S1P by several laboratories including our laboratory [37-44],

Distinct signaling mechanisms of S1PR1, S1PR2 and S1PR3

S1PR1, S1PR2 and S1PR3 are ubiquitously expressed S1P receptor subtypes that are re-
sponsible for mediating diverse actions of S1P in a variety of cell types, through overlapping yet distinctive intracellular signaling mechanisms (Figure 1) [36-47, 48-55 for review]. The expression of the other two S1P receptors S1PR4 and S1PR5 are relatively restricted to the immune and the nervous system, respectively [8].

S1PR1 couples exclusively to G\textsubscript{i} to activate Ras-ERK and PI 3-kinase-Akt/Rac pathways, leading to stimulation of chemotaxis and cell proliferation. S1PR2 couples to multiple G proteins, especially to G\textsubscript{12/13} to induce potent Rho activation, leading to inhibition of Rac and cell migration, and also inhibition of cell proliferation via inhibition of Akt. S1PR3 activates G\textsubscript{q}-PLC-Ca\textsuperscript{2+} pathway, and G\textsubscript{i}-Ras-ERK and -PI 3-kinase-Akt/Rac pathways. S1PR3-G\textsubscript{12/13}–Rho pathway becomes evident only when G\textsubscript{i} is inhibited by pertussis toxin.

Differently from S1PR1, S1PR2 couples to multiple heterotrimeric G proteins, among which G\textsubscript{12/13} coupling to RhoA activation is most prominent [41, 44, 45, 47-59]. S1PR2 exerts, at the site downstream of G\textsubscript{12/13}-RhoA, a potent inhibitory effect on Rac via stimulation of Rac GAP activity, with consequent inhibition of cell migration toward chemotactic growth factors and chemokines [45, 48-59]. S1PR2-mediated, G\textsubscript{12/13}-coupled RhoA activation also exerts potent inhibition of Akt [60, 62], but not ERK, leading to inhibition, rather than stimulation, of cell proliferation.
proliferation [62, 63]. This inhibition of Akt and cell proliferation via S1PR2 is likely achieved by Rho kinase-mediated phosphorylation and activation of PTEN [60-62]. However, S1PR2-mediated inhibition of cell migration is independent of Rho kinase in CHO cells and B16 melanoma cells [45, 56, 59], and is observed in PTEN-deficient glioma cells [13, 64]. S1PR2 also mediates S1P stimulation of PLC and Ca\(^2+\) mobilization via Go, and activation of Ras/ERK and PI 3-kinase pathways via G [41, 45, 47-56].

S1PR3, which also couples to multiple G proteins, potently activates PLC/Ca\(^2+\) signaling pathway via G, in addition to Ras/ERK, PI 3-kinase and Rac via G, mediating mitogenic/prosurvival and chemotactic effects of S1P [42-56, 59]. S1PR3 also couples to G12/13-Rho, although to a lesser extent as compared to S1PR2 [45, 59].

Since S1PR1, S1PR2 and S1PR3 are widely expressed in various types of cells, an integrated outcome of S1P signaling in a given cell type largely depends upon relative expression levels of the S1P receptor subtypes (see below). In addition, ever growing numbers of examples of cross talks between S1P receptor signaling and growth factor or cytokine receptor signaling have been reported. For example, under certain conditions S1PR3 activation leads to activation of TGF\(\beta\) signaling pathway and fibrosis [18, 65]. Update information regarding detailed cross talk mechanisms is available in recently published excellent reviews [8, 12, 13, 66].

**Sphingolipid metabolizing enzymes and S1P transporters**

S1P is generated through phosphorylation of sphingosine by sphingosine kinases SphK1 [67] and SphK2 [68], which share a conserved catalytic domain, but are distinct in other aspects including their structures of non-catalytic domains and expression patterns. S1P is either dephosphorylated by S1P phosphatases (SPPs) [69, 70] to regenerate sphingosine, or degraded by S1P lyase (SPL) to ethanolamine phosphate and hexadecenal [71, 72], the latter reaction serving as the exit from sphingolipid metabolic pathway (Figure 2).

SphKs, SPPs and SPL play important roles not only for production and degradation of S1P but also for controlling cellular levels of sphingolipids including sphingosine and its metabolic precursor ceramide. These sphingolipid species exert growth inhibitory and proapoptotic effects upon elevation of their cellular levels, through multiple mechanisms including activation of protein phosphatases and inhibition of Akt, which leads to activation of apoptotic signaling pathways [73-75].

In the mammalian body, there is a steep S1P concentration gradient across the capillary wall [76, 77]: the plasma S1P concentration is around 500 nM, which is considered to be markedly higher than that in tissue interstitial fluid. The vast majority of plasma S1P derives from red blood cells [78, 79], which have a moderate level of SphK1 but lack S1P degrading enzymes [80], thus function as a huge S1P reservoir and supplier of S1P in blood. Indeed, anemia causes a reduction in the plasma S1P level. Release of S1P from erythrocytes strictly requires acceptor plasma proteins, mostly HDL and albumin [79, 81]. A high concentration of plasma S1P, derived from erythrocytes, is crucial in maintaining vascular integrity, which is achieved by endothelial S1PR1-mediated enforcement of adherence junctions [82, 83]. Endothelial cells and activated platelets also contribute to plasma S1P [84, 85], although to lesser extents. Nevertheless, S1P generated by activated platelets contributes to serum S1P, which is approximately twice as much as that in plasma.

The major part of plasma S1P is bound to HDL (~60%), albumin (~30%) and other plasma proteins, with only a few percentages of total S1P circulating in a free form [15, 79, 81, 86]. It is known that the major determinants of total plasma S1P concentration are the plasma HDL and albumin levels [15]. Many of beneficial effects of HDL, including activation of eNOS, maintenance of endothelial integrity and NO-dependent functions, and protection of cardiomyocytes after ischemia/reperfusion injury, are attributed to HDL-bound S1P [15].

S1P is secreted out of erythrocytes via a transmembrane S1P transporter, whose molecular identity is yet to be established [87]. In a zebrafish mutant miles apart (but not in mammals), deletion of S1PR2 results in an anomaly termed cardia bifida (two primordial heart tissues remaining separated) [88]. Deletion of Spns2, the first physiologically relevant S1P transporter
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Figure 2. Sphingolipid metabolism in various subcellular compartments. Ceramide (Cer) is produced either by de novo synthesis from palmitoyl CoA (palmCoA) and L-serine with sequential enzymatic reactions in endoplasmic reticulum (ER) or through degradation of sphingomyelin (SM) by the action of sphingomyelinases in the plasma membrane and intracellular membranes including lysosomes. Cer is deacylated by ceramidase to yield sphingosine (Sph), which is then phosphorylated by SphK1/2 to generate S1P. S1P is exported through a plasma membrane S1P transporter, leading to activation of the G protein-coupled S1P receptor subtypes (S1PR1~S1PR5). S1P could be either dephosphorylated by S1P phosphatase1/2 (SPP) back to Sph or degraded to ethanolamine-phosphate (Eth-P) and hexadecenal (hxdcnl) by S1P lyase (SPL) to leave sphingolipid metabolic pathway. SphK1 is present in both cytosolic and membrane-bound fractions, both being enzymatically active. SPPs and SPL are located in ER. Intracellular transfer of Cer from ER to Golgi is facilitated by transfer proteins such as CERT, and both Cer and SM traffic between membrane compartments via vesicular transport [172, 173]. PE, phosphatidylethanolamine.

identified in vertebrates, which belongs to the major facilitator superfamily type transporter, gives rise to the same phenotype [89, 90]. It is not known at present whether erythrocyte S1P transporter is a homologue of Spns2.

S1P in the lymph is produced exclusively by lymphatic endothelial cells [91]. There is an increasing concentration gradient of S1P as follows: lymphoid organ tissue interstitial fluid<lymph<plasma. S1PR1 expressed in lymphocytes mediates their egress from thymus, bone marrow and secondary lymphoid organs into lymph and peripheral blood, thus allowing their recirculation that is necessary for immunosurveillance and adaptive immunity [92-94]. S1PR1 is also implicated in innate immunity [16, 95].

SphK1 knockout (KO) mice were phenotypically normal except for 60% reductions in plasma and serum S1P concentrations as compared to
WT mice, without significant decrease in tissue S1P levels [96]. SphK2KO mice were also phenotypically normal, except that they failed to show lymphopenia after administration of FTY720, which is a potent immunosuppressant that acts after being phosphorylated in vivo to cause retention of lymphocytes in secondary lymphoid organs via downregulation of S1PR1 expressed in lymphocytes (see below) [97]. These results indicate that SphK1 is the major isozyme responsible for production of plasma and serum S1P, and that SphK2 could compensate for SphK1 in its absence, and that SphK2 but not SphK1 is responsible for phosphorylating FTY720.

SphK1\(^{-/-}\)/SphK2\(^{-/-}\) double knockout mice, which have no detectable level of S1P, are embryonic lethal around embryonic day E11.5 with defects in vascular and neural development [98], indicating that S1P is produced exclusively by SphK1 and SphK2 in vivo. It is remarkable that severely SphK-deficient Sphk1\(^{-/-}\)/Sphk2\(^{+/+}\) mice are viable with normal phenotype, except for female infertility due to marked accumulation of sphingosine and dihydrosphingosine in deciduum [99]. S1P levels in uterus and deciduum in Sphk1\(^{-/-}\)/Sphk2\(^{+/+}\) mice were comparable to WT mice.

SPL-null mice show markedly high levels of S1P in tissue and serum with accumulation of ceramide and long chain bases, and also generalized derangements in lipid metabolism, resulting in multi-organ abnormalities with vacuolar changes and inclusion bodies, severe lymphopenia, granulocytosis and monocytosis, leading to early lethality after weaning [100, 101].

**S1PR2 is a chemorepellant receptor whereas S1PR1 and S1PR3 are chemotactic receptors**

Cell migration is a process essential for morphogenesis, angiogenesis, immune surveillance, inflammation, tumor cell invasion and metastasis. It is regulated through receptor-mediated processes in response to a variety of ligands, which are either soluble, bound to extracellular matrix or expressed on cell surface.

One of outstanding biological activities of S1P is the ability to regulate cell migration either negatively or positively, which was first recognized to be apparently cell type-dependent. S1P potently inhibits cell migration in a variety of tumor cells including B16 melanoma, breast cancer, and glioblastoma cells [6, 7, 56, 58, 64, 102, 103], as well as vascular smooth muscle cells [104, 105] and neutrophils [106]. By contrast, S1P induces chemotaxis in vascular endothelial cells [107-109], murine embryonic fibroblasts [110], and T and B lymphocytes [111, 112].

Chinese hamster ovary (CHO) cells are an excellent model for studying mechanism of cell migration [45, 48-50, 55, 59]. They vigorously exhibit stimulation or inhibition of cell migration, depending on stimuli. CHO cells overexpressing either S1PR1 or S1PR3 showed pertussis toxin-sensitive, G\(_i\)- and PI 3-kinase-dependent stimulated cell migration toward S1P, with characteristic bell-shaped dose-response curves in a Boyden chamber assay. In contrast, S1PR2-expressing cells or vector control cells did not show a change in cell migration response to S1P. All of the four cell types showed comparable extents of stimulated cell migration in response to their chemoattractant insulin-like growth factor-1 (IGF-1), which was dependent upon PI 3-kinase but not G\(_i\). Importantly, CHO cells overexpressing S1PR2, but not S1PR1 or S1PR3, showed S1P dose-dependent inhibition of cell migration toward IGF-1 [45]. Rho family GTPases Rac, Rho and Cdc42 stimulate actin polymerization to induce lamellipodia, stress fibers and filopodia, respectively [113]. The chemoattractant receptors S1PR1 and S1PR3 mediated Rac activation, whereas chemorepellent receptor S1PR2 did not. S1PR2 and S1PR3 but not S1PR1 mediated activation of RhoA. S1PR1 ~ S1PR3 did not alter Cdc42 activity. The chemoattractant IGF1 induced activation of Rac but not Rho or Cdc42. S1PR2 but not S1PR1 or S1PR3 inhibited IGF-1-stimulated Rac activation. The expression of either a dominant negative (DN) Rac mutant N\(^{17}\)Rac or DN Cdc42 mutant N\(^{17}\)Cdc42, but not DN Rho mutant N\(^{19}\)RhoA inhibited chemotaxis toward IGF-1. N\(^{17}\)Rac and N\(^{17}\)Cdc42 but not N\(^{19}\)RhoA also inhibited S1PR1- and S1PR3-mediated chemotaxis. Thus, stimulated Rac activity and probably basal Cdc42 activity are required for chemotaxis toward IGF-1 and S1P [45]. We also found that S1PR2-mediated inhibition of Rac activity and cell migration in response to IGF-1 were abolished either by expression of DN N\(^{19}\)Rho, inactivation of Rho by pretreatment with C3 toxin, or interfering of S1PR2-G\(_{12/13}\) coupling by expression of the peptide with a sequence of G\(_{12\alpha}\)C-
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terminus or G13α C-terminus [45, 59]. Rho kinase inhibitors, which completely abolished formation of stress fibers and focal adhesions in response to S1P, failed to prevent S1P inhibition of Rac and cell migration [45, 59]. These results indicate that S1P engagement of S1PR2 results in inhibition of Rac and cell migration at the site downstream of G12/13 and Rho activation but not Rho kinase. Rho-dependent, Rho kinase-independent inhibition of Rac seems to involve stimulation of Rac GTPase activating protein (GAP) [45], but not PTEN (unpublished observation) or inhibition of PI 3-kinase [45]. Interestingly, S1PR3 chemotactic receptor, which couples to both Gα and G12/13, functions as a migration inhibitory receptor just like S1PR2 when Gα is inhibited by pertussis toxin [59].

Consistent with the findings obtained in the CHO cell expression system, S1PR1 was predominantly expressed in HUVECs, in which S1P induced Rac activation and chemotaxis in a pertussis toxin-sensitive manner [105, 107, 114-116]. Enforced expression of S1PR2 in endothelial cells dampened or inhibited the stimulatory effect of S1P on cell migration and tube formation on Matrigel [57, 62]. On the other hand, S1PR2 was abundantly expressed in rat aortic smooth muscle (RASM) cells [34, 105], in which S1P potently stimulated RhoA with concomitant inhibition of Rac and cell migration in response to PDGF [105]. Enforced expression of S1PR1 in RASM cells attenuated S1P inhibition of cell migration [105].

**S1PR2 in tumor cells mediates potent inhibition of cell migration, invasion and metastasis, whereas S1PR1 and S1PR3 mediate their stimulation**

By adopting B16 melanoma cells which abundantly expressed endogenous S1PR2 as a model system, we provided the first evidence that endogenous S1PR2 was responsible for S1P inhibition of cell migration and invasion of Matrigel [56]. B16 cells showed a high basal Rac activity under a serum-deprived condition as compared to CHO cells. S1P dose-dependently and markedly suppressed Rac activity, cell migration and invasion. The inhibitory effects of S1P on Rac and cell migration were completely abolished by an S1PR2-selective antagonist JTE-013. In addition, pretreatment with C3 toxin or the expression of a DN N19Rho, but not Rho kinase inhibitors, abolished S1P inhibition of B16 cell migration, indicating that endogenous S1PR2 mediated inhibition of Rac and cell migration via a Rho-dependent, but Rho kinase-independent manner, just like S1PR2 overexpressing CHO cells [45]. Overexpression of S1PR2 sensitized B16 melanoma cells to S1P-induced inhibition of Rac activity and cell migration [56].

Differently from S1PR2, overexpression of either S1PR1 or S1PR3 in B16 melanoma cells blunted the inhibitory effects of S1P on Rac activity and cell migration with rightward shifts of the dose response curves. In these cell types blockade of endogenous S1PR2 by JTE-013 resulted in S1P dose-dependent stimulation of chemotaxis toward S1P [56].

Importantly, S1PR2 mediates S1P inhibition of hematogenous metastasis of B16 melanoma cells in a S1P dose-dependent manner [58]. Tail vein injection of B16 cells resulted in the formation of metastatic nodules in the lung three weeks later. Pretreatment of B16 cells with S1P for just 5 min prior to intravenous injection potently reduced the number of nodules in a dose-dependent manner, with the maximal 40% inhibition obtained at 10⁻⁷ M S1P, suggesting that the inhibitory mechanism involved an early phase of metastasis process [58]. Indeed, studies with fluorescent dye-labeled B16 cells demonstrated that tumor microembolus formation in pulmonary capillaries after intravenous injection was significantly inhibited when tumor cells were pretreated with S1P (Yamaguchi et al., unpublished observation). The expression of N17Rac in B16 cells, which persisted for at least several days, also potently inhibited metastasis by 80% with a reduction in tumor microembolus formation. The expression of N17Rac also inhibited B16 cell proliferation in vitro, although to a much lesser extent (18% inhibition) as compared to its effect on metastasis, whereas S1P did not show any detectable inhibition on B16 cell proliferation in vitro. The inhibitory effect of S1P on metastasis was also observed (40% inhibition) when it was administered intraperitoneally to mice 30 min before injection of B16 cells and afterward once every day (10 micrograms/day), indicating a possible involvement of host cell S1PR2 and also an inhibitory S1P target late in the metastatic process as well [58]. The combination of S1P pretreatment of B16 cells and systemic S1P administration into
mice resulted in a greater than 60% inhibition.

S1PR2 overexpression markedly sensitized B16 cells to S1P-induced inhibition of hematogenous metastasis, with a maximal 80% inhibition [58]. Conversely, overexpression of S1PR1 in B16 cells resulted in marked enhancement of metastasis by in vitro pretreatment of B16 cells with S1P at a relatively low concentration range [58]. Aggravation of metastasis with S1P pretreatment was also observed for B16 cells overexpressing S1PR3 (Yamaguchi et al., unpublished observation). These composite results provided the first molecular basis for S1PR2-selective agonists and S1PR1- and S1PR3-selective antagonists as promising anti-cancer therapeutics in the future [58].

S1PR2-mediated inhibition of tumor cell migration has also been demonstrated in glioblastoma cells in the presence and absence of PTEN [13, 60, 64]. In gastric cancer cells, which express S1PR2 and S1PR3, S1P inhibition of cell migration is strictly related to a higher endogenous expression level of S1PR2 over S1PR3; when S1PR2 expression level is higher than that of S1PR3, then S1P inhibits cell migration, and vice versa [117]. The stimulatory action on cell migration and invasion of S1PR1 and S1PR3 is also evident for thyroid cancer, ovarian cancer and Wilms tumor cells, when their actions overcome inhibitory effects of S1PR2 [118-120].

**Endothelial S1PR1 mediates angiogenic activity**

In cultured endothelial cells (ECs) S1P induced proangiogenic cellular responses including stimulation of cell proliferation, migration and morphogenic capillary-like tube formation in Matrigel, in pertussis toxin-sensitive manners. S1P enhanced angiogenesis in vivo in corneal micropocket assay and Matrigel plug assay [82, 107-109, 114-116]. In accordance with the original observation [35] that endothelial morphogenic activity was associated with upregulation of S1PR1/EDG1 expression, angiogenic effects of S1P on endothelial cells were mediated via S1PR1. S1PR1-selective antagonist inhibited endothelial cell tube formation in vitro and angiogenesis in vivo [121]. S1PR3 also shows proangiogenic activities [114, 116]. In a mouse model of subcutaneous tumor implantation, host S1PR1 was upregulated in tumor microvessels. Intratumoral injections of S1PR1-specific siRNA every three days into LLC tumors, which itself did not express S1PR1, inhibited tumor angiogenesis and tumor growth in vivo [122]. Tumor angiogenesis and tumor growth were inhibited by FTY720, the functional antagonist for S1PR1 (see below) [123-125].

S1PR1KO mice were embryonic lethal between embryonic days E13.5 and E14.5, not because of defective angiogenesis, but because of defective maturation of blood vessels with impaired mural cell coverage and consequent massive hemorrhage [110]. This phenotype of defective vascular maturation was reproduced in mice with vascular endothelial cell-specific conditional deletion of S1PR1 [126]. The investigation into the interaction between vascular endothelial and mural cells revealed that endothelial S1PR1 was required for Rac-dependent, N-cadherin-mediated adhesion between the two cell types [127]. S1PR1/S1PR2/S1PR3 triple KO mice showed more severe phenotype in vascular formation as compared to S1PR1KO mice [128].

**Endothelial and myeloid S1PR2 mediates anti-angiogenic and tumor suppressive activities**

The observation that S1PR2-selective antagonist JTE-013 [56] markedly stimulated in vivo angiogenesis in Matrigel plug assay [57] suggested that S1PR2 might play an inhibitory role in angiogenesis. We created S1PR2KO (S1P2-/-) and S1PR2KO/LacZ knock-in (S1P2LacZ/LacZ) mice to understand in depth the role of host S1PR2 in tumor angiogenesis and tumor growth in vivo [62].

Subcutaneously inoculated Lewis lung carcinoma (LLC) and B16BL6 melanoma grew much more rapidly in S1PR2KO mice as compared to those inoculated in wild type (WT) littermates. LLC tumors in littermate mice that were heterozygous for S1PR2 allele grew at a rate between those of WT and KO mice (unpublished observation). Tumors grown in S1PR2KO mice showed twice as much tumor microvessels as those grown in WT littermates, with enhanced maturity, namely increased mural cell coverage and perfusion in vivo. The numbers of proliferating tumor cells, which were abundant in areas close to tumor microvessels, were also greater in S1PR2KO mice. Analysis on tumor-bearing S1PR2+/LacZ mice demonstrated increased expression of S1PR2 in tumor microvessels, both
Tumor suppressive vs. promoting S1P signaling in endothelial and smooth muscle cells, as well as tumor-infiltrating cells outside of vessels. Lung microvasculature endothelial cells obtained from S1PR2KO and WT littermates showed that S1PR2 in WT endothelial cells mediated inhibition of cell proliferation, migration and morphogenic tube formation. When inoculated into mice together with LLC and B16BL6 cells, S1PR2KO endothelial cells greatly stimulated tumor growth with enhanced angiogenesis as compared to WT endothelial cells. These results indicate that S1PR2 expressed on host endothelial cells inhibits tumor angiogenesis in a cell-autonomous manner.

Accumulating evidence indicates that bone marrow-derived, tumor-infiltrating myeloid cells play crucial roles in tumor development. These cells, including tumor-associated/educated macrophages and CD11b+ population, are responsible for production of proangiogenic growth factors including VEGF-A and activation of matrix metalloproteases (MMPs), which allow liberation of matrix-bound proangiogenic factors and growth factors for tumor cells, and also provides a suitable environment for tumor cell invasion, thus leading to stimulation of angiogenesis, invasion and metastasis [129]. We characterized tumor-infiltrating, S1PR2 expressing cells by bone marrow transplantation experiments with GFP transgenic and S1PR2+/LacZ mice and found that the majority of these cells were bone marrow-derived myeloid lineage cells, which included CD11b+ cell population that expressed S1PR2, by using double staining for β-galactosidase activity and CD11b immunoreactivity. Bone marrow-derived S1PR2 expressing cells were not detected in the tumor microvessel wall, neither in endothelial nor smooth muscle layer. As compared to tumors grown in WT mice that had received bone marrow transplantation from S1PR2WT mice as a donor, tumors grown in WT mice that had received bone marrow transplantation from S1PR2KO mice showed increased expression of VEGF-A, bFGF and TGFβ, and MMP9 activity as compared to WT littermates.

These results indicate that host S1PR2 has a unique tumor-suppressive activity: S1PR2 expressed in bone marrow-derived, tumor-infiltrating myeloid cells and vascular endothelial cells in concert mediates inhibition of tumor angiogenesis and tumor growth [62].

SphK1 could be involved in tumor progression, rather than tumor initiation

Spiegel and colleagues first demonstrated that overexpression of SphK1 in NIH3T3 fibroblasts and HEK293 cells resulted in reduced serum-dependence and increased rate of cell proliferation, with increased survival under serum-deprived condition or against ceramide challenge, implicating the involvement of SphK1 in cancer development [130].

It was then reported that overexpression of SphK1 in NIH3T3 fibroblasts resulted in full transformation with the ability to form tumors in NOD/SCID mice, which led to a proposal that SphK1 was an oncogene capable of inducing tumor initiation [131]. They also showed that phosphorylation of SphK1 at Ser225 by ERK1/2 was required for translocation to the plasma membrane and also for transformation [132, 133]. A mutant SphK1, SphK1S225A, which was enzymatically active but defective in ERK1/2-dependent phosphorylation, failed to show either plasma membrane localization or transformation, whereas the addition of a myristoylation/palmitoylation motif to SphK1S225A conferred constitutive plasma membrane location and recovery of transforming activity in soft agar colony formation [133].

These observations obtained in NIH3T3 cells may not be generalized, however, as in 3T3-L1 cells the overexpression of a myristoylated SphK1 construct, which showed plasma membrane localization, resulted in a potent inhibition, rather than stimulation, of cell proliferation, whereas overexpression of WT SphK1, which distributed in both cytosol and perinuclear membrane structures, did stimulate cell proliferation [134]. Differently from widely accepted concept of oncogene (active mutant) vs. proto-oncogene (WT) paradigm, SphK1 is constitutively active in its WT form, and mutation in...
SphK1 gene or SphK1 gene amplification in cancer has not been reported to date, raising the question whether SphK1 represents an oncogene [13, 135].

Accumulating evidence indicates that SphK1 could be involved in the process of tumor progression, rather than tumor initiation, through multiple mechanisms as discussed below. The mechanisms include amplification of HIF1-dependent responses, paracrine stimulation of endothelial S1PR1-dependent angiogenesis, paracrine/autocrine stimulation of tumor cell invasion, and intracellular roles of SphK1 as a sphingolipid metabolizing enzyme and as a signaling molecule that interacts with an intracellular target.

**Multistep carcinogenesis and hypoxia leads to overexpression and activation of SphK1 in tumor cells**

The expression levels of SphK1 mRNA and protein are elevated in a variety of tumor cells and tumor tissues of mouse and human origin, with positive correlations observed between the SphK1 expression levels and either the extents of resistance to chemotherapeutic agents or radiation, advanced stages or poor prognosis [13, 136-144]. Immunohistochemical analysis shows cytosolic localization of SphK1 protein in SphK1 overexpressing tumor tissues [143, 145]. SphK1 gene amplification in tumors has not been reported thus far. The underlying mechanism for SphK1 overexpression in tumor tissue as compared to normal tissue has not been fully addressed until recently.

Several lines of evidence indicate that either an activating mutation of a proto-oncogene or inactivation of a tumor suppressor gene could lead to SphK1 overexpression. First, the expression of a constitutively active mutant of Ras induces transcriptional upregulation of SphK1 [131]. Similarly, an activating mutation of RET, which is responsible for papillary thyroid carcinoma and multiple endocrine neoplasia (MEN) type 2, also induces upregulation of SphK1 mRNA and protein levels [146]. Second, the expression of v-Src stabilizes SphK1 mRNA, leading to SphK1 mRNA upregulation and protein overexpression [147]. Third, degradation of SphK1 is dependent upon p53 function, and loss of function of p53, which is frequently observed in human cancer, results in reduced rate of SphK1 degradation and upregulation of SphK1 protein level [148]. It is therefore reasonable to postulate that increased expression of SphK1 in tumor cells may be at least in part a consequence of multistep carcinogenesis.

SphK1 overexpression could also result from activation of GPCRs or growth factor receptor/tyrosine kinases. For example, engagement of LPA receptor-1 strongly induces overexpression of SphK1 through a mechanism involving trans-activation of EGF receptor signaling and S1PR3 upregulation [149]. In addition, it is known that engagement of GPCRs and growth factor receptors induce a rapid and transient activation of SphK1 activity [5, 12-14].

Another crucial event that leads to upregulation of SphK1 in tumor cells is hypoxia, which induces SphK1 transcriptional upregulation in a hypoxia inducible factor (HIF)-dependent manner [150, 151]. SphK1 gene has two HIF-responsive elements (HREs) in its promoter region, through which both HIF1α and HIF2α contribute to stimulate SphK1 gene transcription. Indeed, conditioned media from hypoxia-treated tumor cells induced angiogenic responses in human umbilical vein endothelial cells in a S1PR1/S1PR3-dependent manner [150]. In addition to hypoxia-induced transcriptional upregulation of SphK1 gene expression, hypoxia induces stimulation of SphK1 activity upon re-oxygenation through a reactive oxygen species (ROS)-dependent mechanism, which is reported to involve Akt-mediated phosphorylation and functional regulation of GSK3β with downstream signaling [152]. Consistent with this, cardiac hypoxia/reperfusion injury is alleviated in SphK1 transgenic mice [18] and aggravated in SphK1 KO mice, as compared to littermate WT mice [10], and the protective effect of SphK1 is mimicked by S1P and HDL.

Overexpression of SphK1 in tumor cells could enhance tumor angiogenesis and tumor cell invasion through multiple mechanisms involving HIF1 and S1PR1.

It is of note that SphK1, which is upregulated in a HIF1α-dependent mechanism, in turn stabilizes HIF1α [152, 153]. The SphK1-dependent stabilization of HIF1α levels is mediated by the Akt/GSK3β signaling pathway that prevents von Hippel-Lindau protein-mediated proteasomal degradation of SphK1. The pharmacological and
RNA silencing inhibition of SphK1 activity prevents the accumulation of HIF1α and its transcriptional activity [152, 153].

These composite findings strongly suggest that SphK1 and HIF1α constitute a feed-forward amplification loop under hypoxia, resulting in further enhancement of HIF1α-dependent responses, which include increased expression of glucose transporter and glycolytic enzymes for metabolic adaptation to hypoxia, and induction of angiogenic growth factors such as VEGF-A and many others that are involved in tumor progression and metastasis (Figure 3). This amplification loop likely functions in normoxic condition as well, since both SphK1 and HIF1α are upregulated by growth factor stimulation via a Ras-dependent mechanism under normoxia [154]. At present it is not known whether S1P receptors are involved in SphK1-induced, Akt-dependent stabilization of HIF1α. Since Akt is a dominant downstream signaling molecule of G1-coupled receptors S1PR1 and S1PR3, it is likely that tumor-expressing S1PR1/3 participate in the HIF1α-mediated signaling of tumor progression, through autocrine/paracrine stimulation of the receptors.

In addition to HIF1α-dependent production of angiogenic growth factors in SphK1-overexpressing tumor cells, SphK1-catalyzed production of S1P in tumor cells leads to S1P release into cell exterior through a transmembrane S1P transporter, which enables
paracrine/autocrine activation of Gi-coupled S1P receptors, S1PR1 and S1PR3, in both host endothelial cells and tumor cells. Indeed, a recent study by Obeid and colleagues [155] demonstrated for the first time that S1P secreted from SphK1-overexpressing HEK cells and tumor cells induced vascular and lymphatic endothelial cell migration and tube formation in a co-culture system. It is also reported in U-118MG glioblastoma cells that overexpression of S1PR1 and S1PR3, but not S1PR2, resulted in potent activation of urokinase type plasminogen activator (uPA), which is involved in tumor cell invasion [156]. Inhibition of SphK1 by either a pharmacological SphK inhibitor or RNA silencing potently inhibited uPA activation and invasion [156].

Recent studies [123-125, 157] demonstrate that anti-S1P monoclonal antibody and FTY720, both being functional inhibitors of S1PR1 signaling, effectively inhibit angiogenesis and tumor cell proliferation in vivo. These observations strongly suggest that autocrine/paracrine S1P signaling through S1PR1 plays a significant role in these processes.

Overexpression of SphK1 in tumor cells could confer resistance to chemotherapeutics through reduction in ceramide level and potentiation of Akt activity

Overexpression of SphK1 confers not only acceleration of cell proliferation but also resistance to proapoptotic stimuli, which include serum deprivation, ceramide toxicity and chemotherapeutic agents [130]. It is widely recognized that ceramide, which is located upstream of SphK1-catalyzed reaction in the sphingolipid metabolic pathway, accumulates in cells in response to apoptosis-inducing stimuli, such as chemotherapeutic agents and radiation, and mediates cell growth arrest and apoptosis [73, 74, 136, 158]. Underlying mechanisms for ceramide-mediated induction of apoptosis may involve inhibition of Akt activity through enhanced dephosphorylation of Ser473 [159]. It is reported recently by Banno and colleagues [160] that resistance to oxaliplatin was well correlated with high SphK1/2 expression levels and low ceramide level. Oxaliplatin stimulated neutral sphingomyelinase in sensitive cells, resulting in production of ceramide, inhibition of Akt and induction of apoptotic signaling. By contrast, resistant cells showed a high basal Akt activity, which was inhibited by oxaliplatin only when either SphK1 or SphK2 was pharmacologically inhibited or knocked down by RNA interference. Inhibition of Akt was associated with an increase in ceramide synthesis via salvage pathway, followed by apoptosis. These results suggest that SphK1 overexpression confers resistance to oxaliplatin through mechanisms involving suppression of the ceramide level and potentiation of Akt activation [160]. They further provided evidence that oxaliplatin induces upregulation of p53 when SphK1/2 expression and Akt activity were suppressed [160]. Consistent with this, it was previously demonstrated that Akt phosphorylates and activates MDM2, which leads to ubiquitination and degradation of p53 [161], whereas p53 and caspases are involved in degradation of Akt [162].

In addition to ceramide reduction and Akt activation, SphK1 could directly interact with an intracellular target protein to exert anti-apoptotic effect. Upon TNFα stimulation, SphK1 directly interacts with TNF receptor associated factor 2 (TRAF2), leading to SphK1 activation and production of S1P, which are required for NF-κB activation and inhibition of apoptosis [163, 164].

SphK1 deletion could inhibit tumor formation through accumulation of sphingosine rather than reduced S1P production

The inhibition of SphK1 by either RNA silencing or SphK inhibitors leads to inhibition of cell proliferation and induction of apoptosis. The effect of SphK1 deletion on tumor formation was examined in two different mouse tumor models using SphK1KO mice [143, 145]. In both models development of adenoma and adenocarcinoma was inhibited in SphK1KO mice, although underlying mechanism may not be identical.

In azoxymethane- and dextran sulfate-induced colon carcinogenesis model, in which chronic inflammation is involved as a mechanism underlying cancer development, adenocarcinoma lesion in WT mice showed a several fold increase in the SphK1 mRNA expression and two-fold elevation in the S1P level [143]. Adenoma and adenocarcinoma did develop in SphK1KO mice, however, their numbers were both 50% reduced as compared to WT littermates, with reduced numbers of cells in S phase (60%) and increased numbers of apoptotic cells (200%) as
compared to WT tumors [143]. In addition, mRNA levels of inflammation related genes, TNFα and COX2, were elevated in WT cancer tissues by 5 to 10-fold, respectively, whereas in cancer tissues in SphK1KO mice COX2 mRNA level was elevated by only 3-fold and TNFα mRNA was not elevated. The tissue levels of S1P or other sphingolipids in tumor and normal mucosa of SphK1KO mice were not addressed.

In ApcMin/+ mouse model of intestinal adenoma-tous polyposis, polyp lesions showed increases in the levels of S1P and sphingosine, with no change in that of ceramide [145]. Differently from azoxymethane model, ApcMin/+Sphk1−/− mice showed a 50% reduction in polyp size, but not polyp numbers, as compared to ApcMin/+Sphk1+/− mice. Interestingly, the S1P levels in polyp lesion and normal mucosa in ApcMin/+Sphk1−/− mice were comparable to those in ApcMin/+Sphk1+/− mice, respectively, whereas the sphingosine level in tumors of ApcMin/+Sphk1−/− mice was 150% of that in ApcMin/+Sphk1+/+ mice. It was suggested that a marked reduction in proliferating cells in polyps of ApcMin/+Sphk1−/− mice were due to increased level of sphingosine, which was associated with reduced expression of CDK4 and myc; genetic studies indicated that S1PR1 ~ S1PR3 were not likely involved in adenoma development. An increase in the tissue sphingosine level, but not a reduction in the S1P level, was also observed in deciduum of severely SphK-deficient Sphk1−/−Sphk2−/− female mice, which led to lamellar body formation reminiscent of sphingolipidosis, decreased cell proliferation, increased apoptosis and infertility [99].

Further studies are required to fully elucidate molecular mechanisms for SphK1-dependent tumor progression. In addition, generalized overexpression of SphK1 in vivo does not necessarily stimulate tumor formation (our unpublished observations).

**Targeting S1P receptors and SphK1 as anti-cancer treatments**

Targeting the angiogenic and tumor promoting S1P signaling would be promising as an anti-cancer treatment. Anti-S1P monoclonal antibody (Sphingomab) sequesters S1P from binding to cell surface receptors. It is reported to effectively inhibit tumor growth in xenograft and allograft mouse models, through inhibition of tumor angiogenesis, and also through direct inhibitory effects on tumor cells, in which the antibody competed against S1P effects including stimulation of cell proliferation, prevention of apoptosis and production of proangiogenic cytokines [157; 165 for review]. Because the antibody, which specifically binds S1P with a high affinity, is reported to trigger S1P release from erythrocytes [79], which constitute a large reservoir of plasma S1P, the antibody dose that is required to effectively reduce plasma S1P concentration would be high in clinical settings. Delineating suitable antibody delivery system that bypass bloodstream to target primary and metastatic tumor tissue would improve cost performance status. Since anti-S1P antibody should reduce availability of S1P for antiangiogenic and tumor suppressive S1P receptor S1PR2 as well, addition of selective inhibitors of S1PR1 and S1PR3 is expected to potentiate the effectiveness of the antibody in inhibiting angiogenesis and tumor cell migration and invasion.

FTY720, an analogue of sphingosine, acts after SphK2-mediated phosphorylation as an S1P analogue in vivo, with two opposing actions: it could act as an agonist for S1PR1, S1PR3, S1PR4 and S1PR5 but not S1PR2, and also as a functional antagonist for S1PR1. The latter action is due to internalization, ubiquitination and proteasomal degradation of S1PR1 [166]. Thus, FTY720 acts as a potent immunosuppres-sant through downregulation of S1PR1 in lymphocytes to inhibit lymphocyte egress from lymphoid organs and recirculation [91-94, 167]. FTY720 also suppresses angiogenic activity of S1P through downregulation of S1PR1 in endothelial cells [166]. Pretreatment of HUVECs with nanomolar concentrations of FTY720 or FTY720-phosphate downregulated S1PR1 and inhibited endothelial cell migration and Ca2+ mobilization in response to S1P. FTY720 potently inhibited angiogenesis in corneal micropocket assay, subcutaneously implanted chamber assay, and tumor angiogenesis, tumor growth and metastasis in vivo [123-125]. These results are consistent with the notion that S1PR1 expressed in endothelial cells is involved in stimulation of tumor angiogenesis and metastasis. In addition to its inhibitory effect on endothelial cells via downregulation of S1PR1 at nanomolar concentrations, it is reported that micromolar concentrations of FTY720 directly induced apoptosis of a breast cancer cell line [123]. It is also reported in ovarian cancer cells that FTY720 induced
autophagy and necrosis [168]. These observations may be related to recently reported novel actions of FTY720, which include inhibition of SphK1 [169] and autotaxin, the latter being phospholipase D in the plasma responsible for lysophosphatidic acid production [170]. Since FTY720 is a potent immunosuppressant, it is a concern that this effect might reduce immunological anti-tumor activity. Infectious complications should also be carefully watched. Another concern is endothelial dysfunction after prolonged usage of the anti-S1P antibody or FTY720 because S1P maintains vascular barrier integrity and supports eNOS activity via S1PR1 and S1PR3.

Inhibitors of SphK1 for clinical use are currently under intensive investigation [13]. Upon entering bloodstream, SphK1 inhibitors would get into erythrocytes to inhibit SphK1 therein, before being delivered to tumor tissues, which could reduce plasma S1P level to alter vascular barrier integrity and anti-atherogenic effects of HDL. Tumor-directed drug delivery system for SphK1 inhibitors would reduce such adverse effects. Very recently, the first phase I trial of SphK1 inhibitor L-threo-dihydrosphingosine (Safingol) has been conducted [171].

Another promising strategy of anti-cancer treatment that inhibits tumor angiogenesis, tumor growth and metastasis is to potentiate S1PR2 tumor suppressive signaling. A combination of a S1PR2-selective agonist with a S1PR1-selective antagonist [121] is expected to be more potent than either alone.

Conclusion

Recent studies unequivocally demonstrate the existence of the tumor suppressive S1P signaling that is mediated through G12/13-coupled S1PR2 in tumor cells, endothelial cells and bone marrow-derived myeloid cells. S1PR2 negatively regulates tumor angiogenesis, tumor growth and metastasis, counteracting Gi-coupled S1PR1/S1PR3 signaling, which mediates tumor angiogenesis and tumor promotion. Selective activation of S1PR2 in combination with selective blockade of S1PR1/S1PR3 could be an effective anti-cancer therapeutic strategy targeting S1P signaling system, with possibly a reduced side effect.

In tumor cells, hypoxia induces HIF1α-dependent transcription of SphK1 with consequent increases in its mRNA and protein levels, and also its post-translational activation. Oncogenic mutations, including activating Ras mutation and p53 deletion, also contribute to upregulation of SphK1 protein levels and activity. These mechanisms in concert lead to SphK1 overexpression that is occasionally observed in tumor cells. SphK1 thus upregulated in tumor cells in turn stabilizes HIF1α through Akt-dependent regulation of GSK3β, providing tumor cells a feed-forward amplification loop for HIF1α-dependent processes, which include upregulation of angiogenic growth factors, glucose transporters, glycolysis enzymes and many others. SphK1 overexpressed in tumor cells also acts to protect Akt from ceramide-induced deactivation, thus conferring resistance to proapoptotic stresses such as chemotherapeutics and irradiation. These multiple mechanisms, which brings about elevated production and release of S1P and consequent activation of S1PR1 on tumor cells and endothelial cells through autocrine/paracrine mechanisms, in concert lead to tumor promotion, rather than tumor initiation. Significance and precise molecular mechanisms for SphK1 overexpression in tumor biology, as well as S1PR subtype-targeted, selective therapeutic tactics deserve further investigation.

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References


[29] Takuwa N, Iwamoto A, Kumada M, Yamashita K, Takuwa Y. Role of Ca2+ influx in bombesin-
[51] Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine


[77] Hla T, Venkataraman K, Michaud J. The vascular S1P gradient-cellular sources and biological


Tumor suppressive vs. promoting S1P signaling


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