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Review Article

Sphingosine-1-phosphate signaling and cardiac fibrosis

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Sphingosine-1-phosphate (S1P) is a pleiotropic lysophospholipid mediator that acts on 5 members of the G protein-coupled S1P receptor family to induce diverse biological responses. $S1P_1$, $S1P_2$ and $S1P_3$, which are widely expressed receptor subtypes, exert distinct regulatory effects on cytoarchitecture, cell migration, proliferation and gene expression, through differential coupling to heterotrimeric G proteins and downstream signaling, including activation of Rac and Rho small GTPases. Recent studies indicate the involvement of the S1P signaling system in inflammation and fibrosis. Investigations into genetically engineered mice have provided evidence that $S1P_2$ and $S1P_3$ receptors, together with sphingosine kinase 1 (SphK1), which is a major S1P synthesizing enzyme, participate in fibrogenic processes, through mechanisms involving activation of the Rho-dependent pathway and cross-talk with TGFβ signaling. This review will focus on the basics of the S1P signaling system, which include S1P receptor subtype-specific signaling and biological activities, production and degradation of S1P, and currently available *in vivo* data, including our own data, regarding how S1P signaling is involved in cardiac fibrosis, a pathological feature of cardiac remodeling that leads to chronic heart failure, which is one of leading causes of death in developed countries.

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Introduction

Sphingosine-1-phosphate (S1P) is a plasma lysophospholipid mediator with diverse biological activities. Two decades ago S1P was first reported as a mitogenic sphingolipid in cultured fibroblasts[1], and was subsequently recognized to have unique bi-modal regulatory actions on cell migration[2] i.e., acting either as a chemoattractant by itself, or rather as an inhibitor of cell migration toward a chemoattractant.

It is now established that S1P acts through five mem-

bers of the G protein-coupled receptor (GPCR) family for S1P (S1P$_1$ through S1P$_5$), among which S1P$_1$, S1P$_2$ and S1P$_3$ are widely expressed. S1P$_1$～S1P$_3$ receptors not only mediate diverse physiological functions of S1P, which include embryonic vascular formation, maintenance of vascular barrier function, cardiovascular regulation and lymphocyte trafficking, but also play a variety of pathophysiological roles, together with a principal S1P synthesizing enzyme sphingosine kinase 1 (SphK1)[3-22]. Plasma S1P is constitutively produced by SphK1 in red blood cells and vascular endothelial cells in physiological conditions to maintain both vasoprotection and lymphocyte recirculation. In addition, S1P is released from activated platelets, implicating its role in pathophysiology. S1P is also produced by other cell types outside of vessels, such as activated fibroblasts and tumor cells. The latter show upregulation of SphK1 through mechanisms involving hypoxia, p53 inactivation and oncogenic Ras mutation during the course of multistep carcinogenesis, which is implicated in tumor angiogenesis, tumor progression and resistance to chemotherapy and radiation.

Recent studies indicate the involvement of the S1P signaling system in inflammation[8, 13, 14], which provides a profibrotic milieu for fibroblasts to induce their transdifferentiation to myofibroblasts and promotes recruitment of myofibroblast precursors[16]. In addition, investigations into genetically engineered mice have provided evidence that the S1P signaling system represents so far an unidentified participant in fibrogenesis[16], in which S1P$_2$ and S1P$_3$ receptors, together with SphK1 are involved, in close relationship with TGFβ, a well established fibrogenic mediator, and MRTF-A, a major fibrogenic transcription factor acting downstream of Rho.

In this article we first briefly review the basics of the S1P signaling system, which include S1P receptor subtype-specific signaling and biological activities and production and degradation of S1P. We then introduce currently available *in vivo* data regarding how S1P signaling is involved in cardiac fibrosis, which is a common feature of pathological cardiac remodeling of both ischemic and non-ischemic etiology.

More detailed information on the roles for the S1P signaling system in physiology and pathophysiology in general[7, 8, 11-14] and its role particularly in fibrosis in a variety of organs[19-22] are available in recently published review articles and references therein.

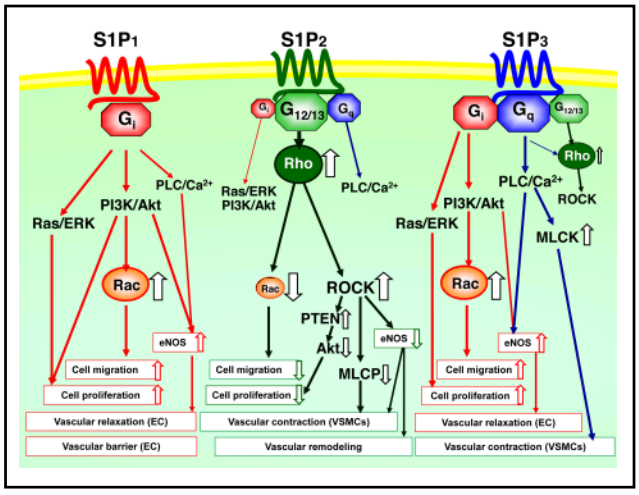

Fig.1 Signaling mechanisms for widely expressed S1P receptor subtypes S1P$_1$, S1P$_2$ and S1P$_3$

S1P$_1$ and S1P$_3$ couple to G$_i$ to induce Rac-dependent chemotaxis toward S1P, whereas S1P$_2$ mediates inhibition of Rac and cell migration through G$_{12/13}$-Rho pathway in a manner independent of ROCK. S1P$_1$ and S1P$_3$ mediate activation of eNOS, whereas S1P$_2$ mediates its inhibition. S1P$_1$ and S1P$_3$ could mediate endothelial cell-dependent vascular relaxation, whereas S1P$_2$ and S1P$_3$ induce vascular smooth muscle contraction in certain vascular beds via G$_{12/13}$ -Rho-ROCK-mediated inhibition of MLCP and G$_q$-Ca^{2+}, calmodulin-dependent activation of MLCK, respectively. Abbreviations used are: ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; ROCK, Rho kinase, PTEN, phosphatase and tensin homolog deleted from chromosome 10 (=phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase); MLCK, myosin light chain kinase; MLCP. myosin light chain phosphatase; eNOS, endothelial nitric oxide synthase; EC, endothelial cells; VSMCs, vascular smooth muscle cells.

S1P receptor subtypes

Signaling mechanisms of widely expressed receptor subtypes, S1P$_1$, S1P$_2$ and S1P$_3$, which mediate the diversity of S1P actions, are summarized in Fig.1. S1P$_1$ couples exclusively to G$_i$, whereas S1P$_2$ and S1P$_3$ couple to multiple G proteins with distinctive efficacies[3]. Downstream of heterotrimeric G proteins the Rho family small GTPases including Rho and Rac are activated[3], which play pivotal and contrasting roles in the regulation of actin cytoskeletal reorganization, cell migration and gene expression. It is of note that Rho activation induces upregulation of fibrogenic genes (see below).

1)S1P$_1$ receptor

S1P$_1$ was first reported as a gene whose expression was upregulated in differentiating ECs, which led to the initial

nomenclature EDG1 that stands for endothelial differentiation gene-1[23].

$S1P_1$ couples exclusively to G_i to activate multiple pathways, which include Ras-ERK, phosphatidylinositol 3-kinase (PI3K)-Akt, PI3K-Rac, and phospholipase C (PLC)-Ca^{2+} mobilization pathways[3, 24, 25]. $S1P_1$ mediates stimulation of cell proliferation through the first two pathways, and acts as a G_i-dependent chemotactic receptor for S1P through Rac activation. $S1P_1$ expressed in lymphocytes are required for their trafficking[26], which is driven by the S1P concentration gradient[15]. S1P facilitates vascular maturation via $S1P_1$, which mediates endothelial cell-cell adhesion and mural cell recruitment. $S1P_1$ knockout (KO) mice are embryonic lethal as $S1P_1$ expressed in endothelial cells is absolutely required for vascular maturation in embryonic stage[27]. Endothelial cell $S1P_1$ is also required for maintenance of vascular integrity after birth, in maintenance of vascular barrier functions with tight assembly of endothelial adherens junctions, and activation of endothelial nitric oxide synthase (eNOS), which requires both binding of Ca^{2+}/calmodulin and Akt-mediated phosphorylation for full activation.

2)$S1P_2$ receptor

$S1P_2$/EDG5/AGR16 was originally cloned in our laboratory as an orphan GPCR highly expressed in vascular smooth muscle cells (VSMCs)[28] and identified as S1P receptor[29] and the first chemorepellant GPCR[30]. $S1P_2$ couples to multiple heterotrimeric G proteins, among which prominent coupling to $G_{12/13}$ leads to activation of Rho and Rho-dependent inhibition of Rac, which is responsible for inhibition of cell migration in the face of concomitant Rac stimulatory signals from either growth factor receptors or G_i-coupled chemotactic receptors[30]. In addition, $S1P_2$ mediates inhibition of cell proliferation through $G_{12/13}$-Rho-Rho kinase (ROCK) - phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and consequent inhibition of Akt[10], which overwhelms mitogenic $S1P_2$-G_i -Ras-ERK pathway. In VSMCs endogenously expressed $S1P_2$ mediates inhibition of cell migration toward platelet-derived growth factor (PDGF)[31] and of recruitment to microvessels and medial layer formation[10]. $S1P_2$-mediated inhibition of cell migration does not require ROCK[30, 32, 33, 44], and is observed in PTEN-deficient tumor cells[34]. $S1P_2$ mediates inhibition of tumor progression through multiple mechanisms, which include direct inhibition of tumor cell migration, invasion and metastasis[33, 35], which are counteracted by overexpression of chemoattractant receptor $S1P_1$ or $S1P_3$[33,35], and inhibition of tumor angiogenesis[10]. $S1P_2$KO mice show enhanced Rac and angiogenic activities in vascular endothelial cells, enhanced recruitment to tumor microenvironment of $CD11b^+$ myeloid cells which are a source of angiogenic cytokines, and aggravation of tumor angiogenesis and tumor progression compared to wild type (WT) littermates[10]. Recent studies demonstrate significance of inhibitory role of $S1P_2$ on cell migration of osteoblasts and osteoclasts in bone morphogenesis and osteoporosis, respectively.

Rho activates two Rho kinase isoforms, ROCK1 and ROCK2, which phosphorylate multiple substrates to induce assembly of actin stress fibers and enhancement of contraction. $S1P_2$ mediates contraction of vascular smooth muscle in certain vascular beds such as coronary and basilar arteries and portal vein, in which $G_{12/13}$-Rho-Rho kinase-mediated inhibition of myosin light chain phosphatase (MLCP) activity is operative[36-40]. A ROCK inhibitor has been approved for treatment of vascular spasm after subarachnoid hemorrhage in Japan, and is recently found by our group to successfully prevent vascular spasm of the bypass grafts *ex vivo* before applying to coronary artery bypass grafting[40].

In the liver, hepatic stellate (Ito) cells, which correspond to pericytes in portal vein branches, transdifferentiate to myofibroblasts in response to a variety of noxious stimuli, which is significantly attenuated in $S1P_2$KO mice, indicating that $S1P_2$ mediates fibrotic signal *in vivo*[12, 19]. In addition, $S1P_2$-mediated contraction of portal vasculature is suggested to contribute at least in part to portal hypertension, as portal vein pressure decreases in response to $S1P_2$-selective antagonist in a bile duct ligation model of liver cirrhosis, in which $S1P_2$ but not other S1P receptor subtypes are strongly upregulated in stellate cells[41].

$S1P_2$KO mice are not embryonic lethal but develop juvenile seizures, deafness and vestibular ataxia of vascular origin, and diffuse B cell lymphoma[42].

3)$S1P_3$ receptor

$S1P_3$ receptor couples to multiple G proteins, mediating activation of G_q-PLC-Ca^{2+}, G_i-Ras-ERK, G_i-PI3K-Akt and G_i-Rac, and also $G_{12/13}$-Rho-ROCK pathways[43]. Similarly to $S1P_1$, $S1P_3$ acts as a G_i-dependent chemotactic receptor for S1P[3, 43]. However, inactivation of G_i by pertussis toxin

unveiled its potential as a $G_{12/13}$-coupled chemo-repellant receptor, just like $S1P_2$[44].

$S1P_3$ mediates either vasorelaxation or contraction, through G_i/G_q-mediated activation of eNOS in endothelial cells and $G_q/G_{12/13}$-mediated phosphorylation of myosin light chain in smooth muscle cells, respectively. In addition, $S1P_3$ mediates negative chronotropic effect in sinoatrial cells through G_i. $S1P_3$KO mice show no obvious abnormality[43], however, they provide evidence that $S1P_3$ is involved in cardiac fibrosis via transactivation of Smad signaling (see below).

4)$S1P_4$ and $S1P_5$ receptors

$S1P_4$ is expressed in the immune and hematopoietic systems, and mediates regulation of immune cell proliferation and cytokine production[7] at high concentrations of S1P as compared to $S1P_1 \sim S1P_3$. $S1P_4$ couples to G_s, G_q, and $G_{12/13}$.

$S1P_5$, which also couples to G_s, G_q, and $G_{12/13}$, is expressed in oligodendroglia, dendritic cells and NK cells, mediating recruitment of NK cells to an inflammatory site[7, 45].

Production and degradation of S1P

S1P is generated exclusively by sphingosine kinases, SphK1 and SphK2, *in vivo* by phosphorylation of sphingosine, which originates from a biomembrane constituents sphingomyelin and other sphingolipids (Fig.2). SphK1 is present in both cytosolic and membrane compartments, whereas SphK2 is localized in endoplasmic reticulum and nucleus. Single knockout mice of neither SphK1 nor SphK2 show any developmental abnormality, except for 60 % reduction in serum and plasma levels of S1P in SphK1KO mice compared to WT mice, however, SphK1/SphK2 double KO mice are embryonic lethal with undetectable level of S1P, showing the same phenotype as $S1P_1$KO mice[46]. In addition to the roles for SphKs in the production of S1P, they play important roles, together with other sphingolipid metabolizing enzymes, in homeostasis of cellular levels of sphingosine and ceramide, the latter being the center of cellular sphingolipid and glycosphingolipid metabolism[8, 9, 13, 14].

S1P is dephosphorylated back to sphingosine by S1P phosphatases (SPP1 and SPP2), or irreversibly cleaved by S1P lyase (SPL) to phosphoethanolamine and hexadecenal at the endoplasmic reticulum (Fig.2). In addition to SPPs and SPL, S1P is degraded at cell surface by lipid phosphate phosphatases (LPP1-3), which show broad substrate specificity not restricted to S1P[8, 9, 13, 14].

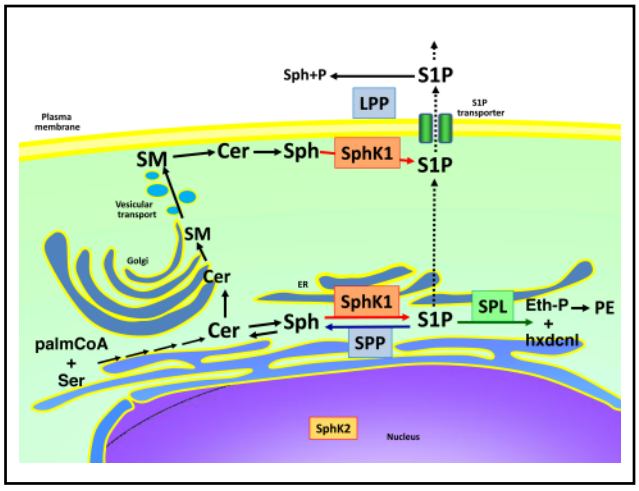

Fig.2 Production, transport and degradation of S1P

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, which are omitted in the figure. Cer is deacylated by ceramidase to yield sphingosine (Sph), which is then phosphorylated by SphK1/2 to generate S1P. SphK1 is present in both cytosolic and membrane-bound fractions, both being enzymatically active. S1P is exported through plasma membrane S1P transporters to outside of cells. Intracellular S1P is either dephosphorylated back to Sph by S1P phosphatases (SPP) or degraded by S1P lyase (SPL) to hexadecenal (hxdcnl) and ethanolamine-phosphate (Eth-P) to leave sphingolipid metabolic pathway, the latter being used for synthesis of phosphatidylethanolamine (PE), the major glycerophospholipid. Extracellular S1P is dephosphorylated by LPP.

S1P is most abundantly present in plasma and then lymph, whereas its concentration in the tissue interstitial fluid is much lower[15]. A steep S1P concentration gradient maintained between these compartments drives $S1P_1$-mediated exit of T and B lymphocytes from secondary lymphoid organs and recirculation, and thus immune surveillance[27]. FTY720, an immunosuppressant approved for treatment of multiple sclerosis, is phosphorylated by SphK2 *in vivo* to form FTY720-phosphate, which can act as an agonist for S1P receptors other than $S1P_2$, but rapidly downregulates $S1P_1$[47]. In effect, FTY720-phosphate acts as a functional $S1P_1$ antagonist, leading to lymphocyte sequestration in secondary lymphoid organs and lymphopenia[47].

Roles for SphKs in physiology and pathophysiology

S1P is constitutively produced in a large amount by SphK1 in red blood cells[27], and also in endothelial cells to a lesser extent, and exported through specific S1P transporter(s) in the cell membrane to plasma[48], in which a total S1P concentration of approximately 1 μM is maintained[15]. The majority of S1P is bound to plasma proteins especially high density lipoprotein (HDL)[49] and albumin, with a high affinity and a high capacity, respectively. S1P bound to HDL induces beneficial vasoprotective effects via endothelial $S1P_1$ (and $S1P_3$)[49, 50], which include maintenance of eNOS activity and tight assembly of endothelial adherens junctions and thus vascular barrier function[4, 12-14, 51].

In addition to erythrocytes and endothelial cells, activated platelets release a large amount of stored S1P[52], which contributes to serum S1P that is higher than plasma S1P. S1P released from activated platelets at the site of vascular injury could contribute to vasoconstriction through either $S1P_2$ or $S1P_3$ expressed in VSMCs, which serves in part as a mechanism for primary hemostasis, and subsequent wound healing through stimulation of cell proliferation in endothelial and other types of cells via $S1P_1$ (and $S1P_3$), as well as pathological processes such as atherogenesis and neointima formation. Indeed, S1P induces upregulation of PDGF in neointima cells via $S1P_1$[53]. Patients with systemic sclerosis show significantly higher levels of serum S1P[54].

A variety of growth factors such as PDGF and proinflammatory cytokines including tumor necrosis factor-α (TNFα) and interleukin (IL)-1β are reported to induce transient activation of SphK[18, 11-14], in which ERK-mediated phosphorylation of SphK1 and subsequent translocation to the plasma membrane is implicated[8, 11-14]. Although SphK1 has a constitutively high catalytic activity, and this ERK-mediated phosphorylation by itself does not further stimulate enzymatic activity, more efficient access to the substrate by membrane translocation of the enzyme would yield higher production of S1P. In the case of TNFα, activation of SphK1 is reported to be involved in COX2 activation and production of prostaglandin E_2[8]. In addition, SphK1 is reported to interact with TNF receptor-associated factor-2 (TRAF2) to mediate anti-apoptotic signaling of TNFα[55].

Importantly, transcriptional upregulation of SphK1 is induced in response to oncogenic Ras and hypoxic stress, the latter being a HIF-1α-dependent process[11, 56]. These and other mechanisms, which include stabilization of SphK1 protein by p53 deletion or Src activation, lead to enhanced expression of SphK1 in tumor cells, especially in late stages of multistep carcinogenesis, which contributes to tumor progression via angiogenic and anti-apoptotic $S1P_1$ (and $S1P_3$) receptor signaling and reduction in the cellular level of proapoptotic sphingolipid, ceramide[57]. However, overexpression of SphK1 is not sufficient for tumor initiation.

With regard to inflammatory signaling, lipopolysaccharide (LPS) acting via toll-like receptor (TLR)-4 induced upregulation of SphK1 at the transcriptional level and enhanced SphK activity in a macrophage cell line[8]. Knockdown of SphK1, however, did not inhibit inflammatory effects of LPS[8]. In other studies SphK1KO mice showed exacerbation, rather than alleviation, of LPS-induced inflammatory responses in experimental models of acute lung injury and neuroinflammation[58].

In a variety of cell types including cultured cardiac fibroblasts, it is reported that TGFβ gradually upregulates SphK1 mRNA and protein levels, resulting in increased production of S1P[59, 60]. The Smad-dependent signaling is reportedly involved in TGFβ-induced upregulation of SphK1 in myoblast to myofibroblast differentiation[61]. siRNA-targeted knockdown of SphK1 blocked TGFβ-induced transcriptional upregulation of target genes, suggesting that SphK1-S1P axis constitutes a part of TGFβ signaling (see below).

As compared to SphK1, the role of SphK2 is less well understood. Recently, it is reported that nuclear S1P produced by SphK2 directly binds and inhibits histone deacetylases 1 and 2 (HDAC1 and HDAC2)[62], which are the first example of intracellular target of S1P to be identified.

Cardiac fibrosis and the SphK1-S1P axis

Cardiac fibrosis is a typical pathological feature that underlies myocardial stiffness and diastolic and systolic dysfunction, atrial fibrillation, and a more advanced clinical condition, i.e., heart failure, which is one of the leading causes of death in developed countries[63]. Cardiac fibrosis constitutes a part of myocardial remodeling in response to a variety of insults imposed on the heart, which include infarction and ischemia/reperfusion injury due to coronary artery disease, biomechanical stress due to hypertension, valvular diseases or congenital cardiac anomalies, and cardiomyopathies due to sarcomere protein gene muta-

tion and other etiologies.

1)Angiotensin II and TGFβ in cardiac fibrosis

Previous studies have demonstrated pivotal roles for local renin-angiotensin-aldosterone system and TGFβ in cardiac remodeling, which consists of cardiomyocyte hypertrophy and interstitial fibrosis[64, 65]. Angiotensin II (AII) stimulation of AT_1 receptor (AT_1R) in cardiomyocytes directly induces activation of both Rac1 and RhoA[66]. Rac1 activation is required for NADPH oxidase-mediated production of reactive oxygen species (ROS)[67], which is a well-established mediator of cardiac remodeling. Rho-ROCK pathway is in part involved in both vascular remodeling[41] and cardiac fibrosis, as evidenced by genetic studies and ROCK inhibitors[68-70]. Studies in ROCK1-deficient mice indicate that ROCK1 is involved in cardiac fibrosis but not hypertrophy in experimental models of either chronic AII infusion or pressure-overload imposed by aortic banding/coarctation[69]. Chronic administration of a specific ROCK inhibitor (which does not discriminate between ROCK1 and ROCK2) suppressed development of fibrosis and also alleviated cardiac hypertrophy, and improved functional parameters in echo cardiography in a pressure-overload model[70], suggesting a possibility that ROCK2 is involved in development of hypertrophy. Consistently, AT_1R blockers (ARB) or angiotensin converting enzyme (ACE) inhibitors, and statins are widely adopted as evidence-based medicines[63, 71], the latter being an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which, in adition to being a rate-limiting enzyme for synthesis of cholesterol, is responsible for synthesis of mevalonic acid and post-translational isoprenylation of Rho and Rac GTPases for their translocation to the plasma membrane and subsequent activation.

AT_1R activation induces upregulation of TGFβ expression in both cardiomyocytes and fibroblasts[72]. Cardiac remodeling due to either chronic AII infusion or sarcomere protein mutation is mediated by TGFβ action, as evidenced by inhibition in TGFβ1-KO mice or with anti-TGFβ antibody[64, 65].

2)TGFβ and Rho

TGFβ is essentially required for diverse aspects of development, physiology and also pathophysiology, including cardiac remodeling. TGFβ plays a central role in inducing transdifferentiation of fibroblasts to myofibroblasts, which are responsible for massive production of extracellular matrix (ECM) components such as collagen type I/III and fibronectin, with increased capacity in cell migration and proliferation and with smooth muscle phenotype such as expression of smooth muscle α-actin (αSMA) and contractility. TGFβ receptor engagement leads to serine/threonine phosphorylation of Smad2/3, which after complex formation with Smad4 is recruited to promoter regions of target genes such as connective tissue growth factor (CTGF) and plasminogen activator inhibitor-1 (PAI-1) to induce their transcriptional upregulation, leading to promotion of fibrogenesis and tissue remodeling. An expanding pool of myofibroblasts in turn produces TGFβ, further promoting cardiac remodeling and dysfunction in a vicious feed-forward mechanism.

In addition to the canonical Smad-dependent pathway, TGFβ activates a number of non-Smad pathways, among which is the Rho-dependent pathway. Studies in cultured cells have demonstrated that TGFβ could induce a biphasic activation of RhoA and stress fiber formation with various time courses[73-75]. In murine embryonic fibroblasts (MEFs) TGFβ induces a rapid formation of stress fibers that is maximal after 15 min and is sustained for at least 24 h[75]. In other types of cells such as Swiss 3T3 fibroblasts[75] and epithelial cells[73-76] stress fibers gradually develop for hours and days in a manner dependent on protein synthesis[76], with stress fibers consisting of not only phalloidon-stained F-actin but also αSMA, phosphorylated form of MLC and other associated proteins, and could be maintained with even basal activity of RhoA[74, 75]. In cultured cells of epithelial origin, TGFβ induces a well characterized epithelial to mesenchymal transition (EMT), in which E-cadherin and adherens junctions are downregulated, whereas N-cadherin, αSMA, and other genes that are related to the smooth muscle phenotype are upregulated. Recent evidence indicates that Rho- and ROCK-dependent activation of myocardin-related transcription factor A (MRTF-A, also called MAL or MKL-1) is involved in TGFβ-induced EMT and fibrogenic signal transduction. TGFβ induces ROCK-dependent polymerization of actin stress fibers, resulting in a reduction in cytoplasmic G-actin concentration and consequent nuclear translocation of MRTF-A, which has been sequestered in the cytoplasm by binding of G actin to nuclear localization signal. In an EMT model system of MDCK kidney epithelial cells MRTF-A forms a complex with activated Smad3 in response to TGFβ, and via

Smad3 the complex binds to a specific GCCG-like motif in the promoter region of genes that are involved in down-regulation of E-cadherin and the adherens junction[76]. In addition, MRTFs upregulate actin cytoskeletal proteins including β-actin and αSMA via interaction with serum response factor (SRF)[76]. EMT is abolished by inactivation of RhoA and by expression of a dominant negative mutant of MRTF-A[76].

Recent *in vivo* evidence indicates that MRTF-A is involved in cardiac fibrosis[77]. MRTF-A KO mice show reduced scar formation after myocardial infarction or in response to chronic infusion of AII, with reduced expression of αSMA and a number of ECM proteins including several types of collagen[77]. SRF/MRTF-A complex binds via SRF to a CArG box in serum response element (SRE) of the promoter region of *collagen1a2*, resulting in its transcriptional upregulation[77].

The mechanism underlying TGFβ-induced activation of Rho is not fully elucidated. In cultured epithelial and endothelilal cells Smad-dependent upregulation of Rho GDP/GTP exchange factors (RhoGEFs), such as NET1 and Arhgef5, has been reported[78]. However, this mechanism, which takes several hours, does not explain reported time courses of Rho activation[73-75]. Multiple mechanisms are likely to be involved in TGFβ-induced activation of Rho.

3)SphK1-S1P axis and TGFβ

Cardiomyocytes and cardiac fibroblasts express SphK1 and $S1P_1 \sim S1P_3$ receptors, with the most abundant subtype being $S1P_1$ and $S1P_3$, respectively[79].

Initially the S1P signaling system attracted attention in the field of cardiovascular research because of its favorable role in protection against acute myocardial ischemia/reperfusion injury[80]. Genetic studies have demonstrated that $S1P_2$ and $S1P_3$ are involved in mediating this favorable effect of S1P and HDL, which is a major blood carrier protein for S1P, and that endogenous S1P is cardioprotective[80].

In sharp contrast to protective effect of S1P signaling against acute ischemia/reperfusion, excess S1P signaling in the myocardium for a prolonged period of time exerts deleterious effect[18]. We have generated transgenic (TG) mice that overexpress SphK1 under a universal promoter. SphK1TG mice grew up normally with normal blood pressure, blood cell counts and blood biochemistry. Plasma and serum S1P levels were comparable to those in wild type mice. SphK activity and S1P level in transgenic heart tissues showed approximately 20-fold and 1.7-fold increases, respectively, compared to WT littermates, with normal sphingosine and ceramide levels. Consistent with the notion that SphK1 plays a cardioprotective role against ischemia/reperfusion injury, SphK1TG mice showed 30% reduction in infarct size. However, SphK1TG mice spontaneously developed cardiomyocyte degeneration and fibrosis without hypertrophy, which started by 12 weeks and aggravated with age[18]. SphK1TG mice showed increases in the active forms of RhoA and Rac1 small GTPases in cardiac tissues and elevated oxidative stress markers. Administration in juvenile mice of a statin or an anti-oxidant prevented development of fibrosis. By contrast, a hypotensive dose of an AT_1 angiotensin II receptor blocker was without any effect. In addition, SphK1TG mice showed increased phosphorylation of Smad2/3 in the heart tissue compared to WT mice, without increase in TGFβ mRNA or protein levels. In $S1P_3$KO genetic background, cardiac fibrosis in SphK1TG mice was greatly attenuated and Smad2/3 phosphorylation was significantly reduced. These results indicate that persistent activation of SphK1 in the heart leads to cardiac fibrosis through multiple mechanisms, including Rac and Rho activation, ROS, and transactivation of TGFβ signaling pathway[18]. The mechanism for this transactivation is not known at present.

It is reported in cultured adult murine cardiac fibroblasts that S1P stimulates their transdifferentiation to myofibroblasts in $S1P_2$- and ROCK-dependent manners[60]. In addition, TGFβ upregulated the expression and activity of SphK1, and TGFβ-stimulated collagen production was inhibited by an anti-S1P monoclonal antibody and SphK1 or $S1P_2$ siRNA, suggesting that the effect of TGFβ is mediated by SphK1 and autocrine/paracrine activation of $S1P_2$[60]. In our laboratory, crossing of the SphK1-transgenic mice with $S1P_2$-null mice failed to produce double mutant mice. It deserves further investigation to elucidate *in vivo* contribution of $S1P_2$ in cardiac fibrosis.

In another recent study using mouse cardiac fibroblasts it is shown that apelin, an adipocyte-derived cardioprotective peptide that acts on GPCR, inhibits SphK1 activity and transdifferentiation to myofibroblasts in response to TGFβ[81]. Knockdown or pharmacological inhibition of SphK1 also inhibited myofibroblast transdifferentiation. Moreover, *in vivo* administration of apelin attenuated endogenous SphK1 activity in myocardium and the develop-

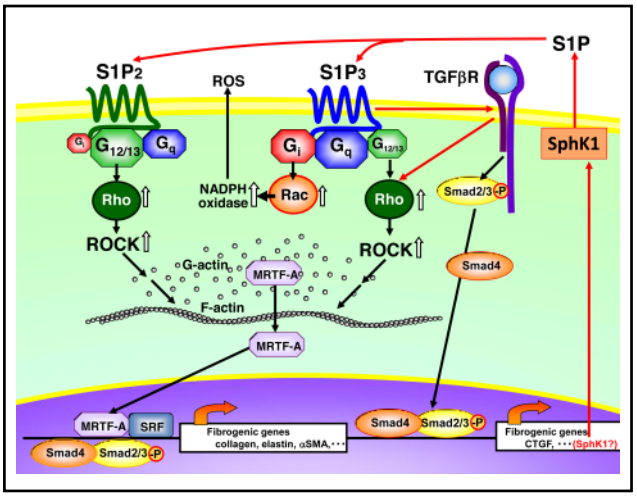

Fig.3 Cross-talk mechanisms between TGFβ signaling and the SphK1-S1P axis

TGFβ receptor engagement activates Smad-dependent canonical signaling in myofibroblasts to induce transcriptional upregulation of connective tissure growth factor (CTGF) and other fibrogenic genes. This pathway also reportedly upregulates SphK1 mRNA and protein levels, leading to local increase in S1P concentration, which in turn acts on $S1P_2$/$S1P_3$ receptors in an autocrine/paracrine manner to activate $G_{12/13}$-Rho-ROCK pathway. Rho-ROCK-dependent polymerization of actin stress fibers leads to liberation of myocardin-related transcription factor-A (MRTF-A) from G-actin-mediated cytoplasmic sequestration and subsequent nuclear translocation of serum response factor (SRF)/MRTF-A complex, which has been shown to induce transcriptional upregulation of a subset of fibrogenic genes including *collagen 1a2* through a conserved CArG element in the promoter region, which is located within Smad3- and Sp1-dependent promoter region. Thus, the SphK1-S1P axis could constitute a part of TGFβ-dependent activation of Rho-ROCK pathway, In addition to Rho-ROCK pathway, $S1P_3$ —mediated activation of Rac leads to generation of reactive oxygen species (ROS). Moreover, $S1P_3$ activation leads to Smad2/3 phosphorylation *in vivo* and *in vitro*.

ment of myocardial fibrosis in response to pressure-overload by aortic banding-coarctation[81]. Apelin also protects against development of cardiac fibrosis in response to chronic AII administration[82].

In our model of SphK1TG mice, $S1P_3$ receptor-mediated transactivation of Smad-dependent pathway and $S1P_2$/$S1P_3$-mediated Rho-ROCK pathway likely converge at upregulation of fibrogenic genes in cardiac fibroblasts (Fig.3). It is conceivable to postulate that under pro-fibrotic conditions where TGFβ is increased, SphK1 is upregulated to locally produce S1P, which in turn acts on $S1P_2$/$S1P_3$ receptors in an autocrine/paracrine manner to amplify activation of ROCK1. ROCK-dependent nuclear translocation of SRF/MRTF-A results in transcriptional upregulation of fibrogenic genes in concert with Smad-dependent activation. Previous studies in cultured cell system are consistent with this notion[21, 83].

Conclusion and future perspectives

The S1P signaling system, including the major S1P synthesizing enzyme, SphK1, and widely expressed major S1P receptor subtypes, $S1P_1$, $S1P_2$ and $S1P_3$, plays diverse physiological and pathophysiological roles, through regulation of actin cytoskeletal reorganization, cell migration, proliferation, survival and differentiation. $S1P_2$ and $S1P_3$ are involved in fibrogenic signaling through $G_{12/13}$-Rho-ROCK pathway with downstream transcriptional upregulation of genes that are directly involved in myofibroblastic differentiation and production of ECM proteins such as collagen. The SphK1-$S1P_2$/$S1P_3$ axis could be activated at the site downstream of TGFβ action. On the other hand, transactivation of Smad signaling pathway by $S1P_3$ also serve to potentiate fibrogenic signaling, although the underlying mechanism remains elusive. By contrast, $S1P_1$ in vascular endothelium acts to protect against inflammatory cell extravasation via re-enforcement of adherens junction assembly and vascular barrier function, and also mediates eNOS activation, which is anti-fibrogenic, together with endothelial cell $S1P_3$. $S1P_1$ and $S1P_3$ expressed in inflammatory cells and fibroblasts mediate their migration toward S1P, which could be locally produced in fibrogenic milieu through activation of SphK1, whereas $S1P_2$ rather mediates inhibition of inflammatory cell recruitment. Recent studies demonstrate increased S1P levels in serum of patients with systemic sclerosis[54], which could be derived from activated platelets to contribute to peripheral vasoconstriction and Raynaud's phenomenon and vasculopathies. Patients with idiopathic pulmonary fibrosis also show increased levels of S1P in serum and bronchoalveolar lavage fluid[84]. The SphK1-$S1P_2$/$S1P_3$ -Rho-ROCK axis, which constitutes a part of TGFβ signaling system, would become novel therapeutic targets to alleviate fibrotic diseases and organ dysfunction.

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Conflict of Interest

The authors declare no conflict of interest.

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