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석사학위 논문

A study on the role of sphingosine kinase 1 in hepatic stellate cell activation

조선대학교 대학원

약 학 과

백 진 솔

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스핑고신 키나아제 1 조절을 통한
간성상세포 활성화 제어 연구

2023 년 2 월 24 일

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이 논문을 약학 석사학위신청 논문으로 제출함

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ABBREVIATIONS

ActD	Actinomycin-D
TGF-β	transforming growth factor-β
α-SMA	alpha-smooth muscle actin
ECM	extracellular matrix
PAI-1	plasminogen activator inhibitor type 1
SphK	sphingosine kinase
S1P	sphingosine-1-phosphate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SBE	Smad binding element
AP-1	activator protein 1
EGCG	epigallocatechin gallate
ALB	albumin
HSCs	Hepatic stellate cells

국 문 초 록

스핑고신 키나아제 1 조절을 통한
간성상세포 활성화 제어 연구

백 진 솔

지 도 교 수: 기 성 환

약학과

조선대학교 대학원

간섬유화는 간염 바이러스, 과도한 알코올 섭취, 비알코올성 지방간 등 다양한 병인에 의한 상처치유 반응으로 인한 임상적 후유증으로 간주되며 간 섬유화가 지속될 시 간경변으로 진행될 수 있다. 간 손상 시, 간은 간 기능 및 구조를 방해하는 세포외 기질(ECM)의 축적 및 간성상세포(hepatic stellate cell)의 활성화를 촉진하며, 간성상세포는 비타민 A 함유 지질 방울이 있는 휴지기 상태에서 근섬유아세포로 분화되며 표현형 변화가 일어난다. 이러한 간섬유증의 진행에 있어서 간성상세포가 중요한 역할을 함이 잘 보고되어 있지만, 간성상세포 활성화 및 섬유화 진행 억제를 위한 새로운 조절분자 및 관련 메커니즘 발굴 연구는 여전히 필요하다. 스핑고신 키나아제 1(Sphingosine kinase1, SphK1)는 세포 성장, 증식 및 분화를 비롯하여 수많은 생리활성을 조절하는 물질이다. SphK1/S1P/S1PRs 축이 간

섬유화 발병 및 진행과 관련된다는 보고가 있지만 간성상세포 활성화 과정에서의 스핑고신 키나아제 1 의 발현 및 기능에 대한 연구는 제한적이다. 본 연구에서 우리는 간성상세포 활성화 과정에서 스핑고신 키나아제 발현이 증가되는지, 그렇다면 간성상세포 활성화에 미치는 영향과 관련 메커니즘을 규명하고자 하였다. 우리는 간경변증 환자 및 사염화탄소 유도 마우스의 간 조직 면역염색 샘플에서 스핑고신 키나아제 1 의 발현이 증가되어 있음을 확인하였다. 또한 사염화탄소 및 담관 결찰 마우스 간섬유화 동물 간조직에서 증가된 스핑고신 키나아제 1 발현을 관찰하였다. 스핑고신 키나아제 1 은 실질세포인 간세포보다 간성상세포에서 더 높게 발현되었으며 간성상 세포주인 LX-2 세포에 TGF-β를 처리하였을 때 스핑고신 키나아제 1 의 발현이 증가됨을 확인하였다. 이러한 결과는 스핑고신 키나아제 1 발현이 간섬유화 진행과정에서 증가되어 간섬유화 진행에 관여함을 시사한다. 다음으로 우리는 TGF-β에 의한 스핑고신 키나아제 1 의 발현증가가 Smad3 의존적임을 규명하였다. 나아가 스핑고신 키나아제 1 의 knockdown 및 과발현을 통하여 TGF-β에 의한 간섬유화 유전자 발현 증가에 있어서 스핑고신 키나아제 1 의 역할을 증명하였다. 스핑고신 키나아제 1 에 의해 매개되는 간섬유화 관련 유전자 발현 증가는 AP-1 활성화에 의존적이었으며, Smad 신호와는 무관하였다. 마지막으로 우리는 스핑고신 키나아제 1 조절을 통한 간섬유화 제어 후보약물로 녹차에 주성분인 폴리페놀 중 하나인 epigallocatechin gallate(EGCG)을 발굴하였다. EGCG 는 TGF-β에 의한 스핑고신 키나아제 1 의 발현 및 TGF-β에 의해 증가되는 간섬유화 유전자 발현을 억제하였다. 종합적으로 TGF-β에 의한 스핑고신 키나아제 1 발현 증가는 AP-1 활성화를 통해 간섬유화를 촉진하고 EGCG 는 SphK1 억제를 통해 TGF-β에 의한 간성상세포 활성화 및 간섬유화 억제에 기여할 수 있음을 제시한다.

I. INTRODUCTION

Liver fibrosis is considered as a clinical sequel caused by the wound healing response to various etiologies such as hepatitis virus, excessive alcohol intake, and nonalcoholic fatty liver, can progress to cirrhosis which are princeps medical problem with high morbidity [1]. Upon liver injury, the liver promotes accumulation of extracellular matrix (ECM), which disturbs liver function and architecture, and activation of hepatic stellate cells (HSCs) [2, 3]. HSCs are the primary cell type for disproportionate ECM disposition, and undergo phenotypic change from quiescent with vitamin A-containing lipid droplet to proliferative myofibroblast cells [4]. Until now, there is little information on the clinically effective way to inhibit HSC activation and fibrosis progression. Therefore, identification of the regulatory molecules and related mechanisms connected with this process is still necessary to develop new diagnostic and therapeutic targets related to liver fibrosis.

During phenotypical transdifferentiation of HSCs, transforming growth factor-β (TGF-β) exerts the most potent profibrogenic and fibroproliferative cytokine by paracrine and autocrine manner [3] [5]. TGF-β-driven activation of transmembrane receptors, composed of type I and II, transactivates Smad proteins or Smad-independent proteins including mitogen activated protein kinase (MAPK) (i.e., extracellular signaling kinase (ERK), p38, and c-jun N-terminal kinase (JNK)[6-8]. These TGF-β-activating signaling pathways stimulate induction of ECM genes such as collagen, plasminogen activator inhibitor type 1 (PAI-1) and α-smooth muscle action (α-SMA) [9, 10].

Because of dramatic changes in the microenvironment of the tissue upon HSC activation, multiple intra/extracellular ligands and fibrogenic stimuli besides TGF-β are elevated [11-13]. Increased attention has been paid to sphingosine-1-phosphate (S1P), a bioactive lipid acting as a key mediator of numerous liver pathophysiological responses such as

cell proliferation, differentiation, migration and angiogenesis and functions as an intracellular second messenger, and its related molecules and pathways [14-16]. S1P, a ligand for G-protein-coupled receptors termed S1P receptors (S1PRs), is converted from sphingosine by two sphingosine kinases (SphKs), SphK1 and SphK2 [17]. It has been demonstrated that the significance of SphK1/S1P/S1PRs axis are implicated in liver fibrosis [18]. Because SphKs converge signals from sphingosine and generate S1P-mediated signaling cascade, the level of SphKs may exert profound effect on the amplification or blunting of physiological effect on liver fibrosis. Until now, little is available on the regulatory mechanism and the role of SphKs on liver fibrosis.

Here, we scrutinized whether expression of SphKs in HSCs was modulated by TGF-β signaling, and if so, what the sequential impacts on HSC activation that finally leads to liver fibrosis and how it is regulated. Observance of SphK1 in patient with cirrhotic liver proved the clinical significance of SphK1 on the development of liver fibrosis. In addition, evidence for the expression and role of SphK1 on HSC biology were demonstrated in fibrotic animal models and *in vitro* cell models. We also found that SphK1 was plentifully expressed in HSCs as compared with hepatocytes, which was upregulated by TGF-β treatment via Smad signaling. Moreover, modulation of SphK1 changed TGF-β-mediated activation of MAPKs, but not Smad phosphorylation. It can be discovered that SphK1-related downstream signaling and its effects were relied on S1PR. Interestingly, we identified that epigallocatechin gallate (EGCG), one of the most plentiful polyphenols in green tea, has anti-fibrogenic effect via blunting TGF-β-mediated SphK1 expression. Our findings illustrated the novel regulatory mechanism and role of SphK1 in HSC activation and further extends biochemical and physiological basis on liver fibrosis.

II. MATERIALS AND METHODS

1. Materials

SphK1 and Sphk2 antibody was purchased from Proteintech (Rosemount, IL, USA). Phospho-JNK1/2, JNK1/2, phospho-ERK, ERK, phospho-p38, p-38 antibodies were provided through Cell Signaling (Danvers, Massachusetts, USA). Fibronectin and Plasminogen activator inhibitor-1 (PAI-1) and antibodies were supplied by BD Bioscience (Mountain View, CA, USA). Actinomycin-D (ActD), Epigallocatechin gallate (EGCG) , alpha-Smooth muscle actin(α-SMA) and β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). VPC23019 was from Cayman (Ann Arbor, MI, USA). TGF-β was purchased from R&D Systems (Minneapolis, MI, USA). Secondary antibodies anti-rabbit or anti-mouse were provided by Invitrogen (Carlsbad, CA, USA).

2. Cell culture

Propagation of human immortalized HSCs (LX-2) was generously obtained by Dr. S.L. Friedmann (Mount Sinai School of Medicine, NY, USA). Rat hepatic stellate cell line (HSC-T6) was purchased from Merck Millipore (Darmstadt, Germany). Cell cultured in DMEM with 50 U/mL penicillin/streptomycin, 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, USA). All cell lines were retained at 37℃ in a humidified 5% CO2 atmosphere.

3. Primary hepatic stellate cells and Hepatocytes isolation

ICR mice were purchased from Oriental Bio (Sungnam, South Korea). Separation of hepatic stellate cells and hepatocytes was performed as follows. First, a catheter was intubated into the hepatic portal vein, followed by in situ perfusion with Ca^{2+}-free HBSS for 20 min at 37℃, and then followed by perfusion with a solution containing 0.05% collagenase and Ca^{2+} for 20 min.

After tear off the liver, filter use 70 μM cell strainer (BD Bioscience), and isolate hepatocyte by centrifuged 3 times for 2 m at 50 x g. Primary HSCs were isolated according to the previously published method [19]. Quiescent HSCs were cultured for 0 day and activated HSCs were cultured for 7 days.

4. Cell viability assay

To evaluate cytotoxicity, LX-2 cells seeding plated in a 12 well plate, treated with EGCG for 12 h, and then stained with MTT (dissolved in MEM medium at a concentration of 0.1 mg/mL for 20 m). The medium was then removed from the well, and the formazan crystals in the well were dissolved by adding 400 μL of DMSO. The absorbance of each sample was measured at 550 nm and measured using a microplate reader (SpectraMAX, Molecular Devices, Sunnyvale, CA, USA). Cell viability was defined relative to control.

5. Immunoblot analysis

SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to previously performed procedures [20]. After harvesting the cells, they were centrifuged at 3,000 x g for 3 min and lysed for 1 h by adding RIPA buffer. The lysate was centrifuged (12,000 × g for 10 min) to obtain a supernatant, and proteins were extracted. Protein samples were subjected to SDS-PAGE and transferred to a nitrocellulose membranes. The membranes were blocked with 5% skim milk for 0.5 h and washed 3 times at 10 minute intervals then cultivated with primary antibody overnight at 4°C. The next day, they were cultivated with peroxidase-conjugated secondary antibody for 1 h Immunoreactive proteins were visualized with the ECL chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA).

6. RNA isolation and RT-PCR analysis

Cells were lysed using TRIzol (Invitrogen, Carlsbad, CA, USA). To obtain total RAN,

chloroform was added to the cells dissolved in trizol, and the sample was centrifuged at 12,000 x g for 20 min. The supernatant was shifted to a new tube, mixed with an equal volume of isopropanol and centrifuged at 12000 x g for 20 min to precipitate RNA. To obtain cDNA, total RNA (2 μg) was reverse transcribed using oligo (dT) 16 primers. The cDNA was amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejeon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA, USA). PCR products were analyzed by gel electrophoresis (2% agarose in 0.5X TAE buffer), stained with ethidium bromide (Sigma, St. Louis, MO, USA), and visualized in gel documentation system (Fujifilm, Tokyo, Japan). The primer sequences used are as follows: human SphK1 sense 5'-CTTCCTTGAACCATTATGC-3', and antisense 5'-CCGATACTTCTCACTCTC-3'; human SphK2 sense 5'-TTTCCGGAAGAAAGGGATCT-3', and antisense 5'-TAGGCTGGTAGGAGCAAGGA-3'; human GAPDH sense 5'-GAAGGTGAAGGTCGGAGTC-3, and antisense 5'-GAAGATGGTGATGGGATTTC-3'. GAPDH was used as a reference gene for normalization.

7. Wound-healing assay

Cell migration was excuted using Culture-Inserts (ibidi culture-insert 4wells, ibidi GmbH, Martinsried, Germany). LX-2 cells were seeded at a density of 3 x 10^5 per well, and culture inserts were removed when cells reached 80-90% confluence. Then, the cells were washed with PBS to remove floating cells, and the medium was changed to MEM media containing 0.1% FBS. Cells were treated with EGCG (10, 30 μM) in the presence or absence of TGF-β (2 ng/mL) ,and Photomicrographs were taken at 0 and 12 h at 50x magnification using a camera attached to a microscope (Axiovert 200 M, Carl ZEISS, Germany).

8. Luciferase assay

Luciferase activity was measured LX-2 cells were seeded in 12-well plates, serum-starved for 3

h, and Smad binding element (SBE)-Luc, activator protein 1 (AP-1)-Luc and pRL-TK plasmids in the presence of Lipofectamine® 2000 Reagent (Invitrogen, San Diego, CA, USA) for 3 h. And then, transfected cells were allowed to regain in MEM containing 1% fetal bovine serum (FBS, Atlas Biological, Fort Colins, CO, USA) overnight and treated with EGCG (10, 30 μM) in the presence or absence of TGF-β (1 ng/mL). Luciferase activity was measured in cell lysates using a dual luciferase assay system (Promega).

9. siRNA knockdown and Transient transfection experiment

LX-2 cells were transfected with comparative control siRNA (100 pmol) or siRNA against SphK1 (100 pmol; Dharmacon, Lafayette, Colorado) for 24h. Then, they were allowed to regain overnight in MEM containing 1% FBS and then treated with TGF-β for 12 h. SphK1 plasmid were obtained from Addgene (Watertown, Massachusetts). The transfection was performed by pcDNA (MOCK) or pcDNA-SphK1 WT (SphK1) for 24 h using Lipofectamine® 2000 Reagent (Invitrogen, San Diego, CA, USA) in accordance with the manufacture's protocols.

10. Animal experiments

A 6 weeks old male ICR mice was purchased from Oriental Bio (Seongnam, Korea) and acclimated for 1 weeks. To induce liver fibrosis, CCl4 (0.5 mg/kg; dissolved in 10% olive oil) was injected intraperitoneally into mice three times a week for 2 weeks, and the common bile duct was exposed near the duodenal attachment and ligated with 6.0 silk. A sham control group was similarly subjected to the operation without BDL. Animal experiments were conducted with the approval of the Animal Care and Use Committee of Chosun University.

11. Patient samples

Liver samples were provided by Chosun University Hospital in Korea. It was collected from 10

cancer patients diagnosed presence or absence of cirrhosis through histological examination and ultrasonography and was recognized by Institutional Review Board of Chosun Medical Center (No. 2013-04-005).

12. Confocal microscopy

Liver tissue were stationary in 4% paraformaldehyde and permeabilization with Triton X-100. Tissues were immunostained with SphK1 antibody overnight and then cultivated with goat anti-rabbit IgG (Invitrogen). Tissue sections were treated overnight with antibodies against SphK1 and desmin or α-SMA for 3h. Next, coverslipped with mounting medium including DAPI (Golden Bridge International, California). For sample detection, a laser-scanning confocal microscope (A1; Nikon Instruments Inc., Melville, New York) was used.

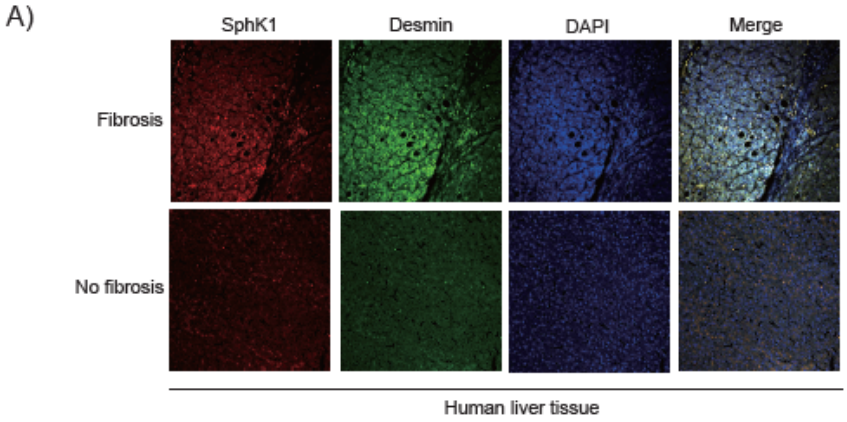
13. Statistical analysis

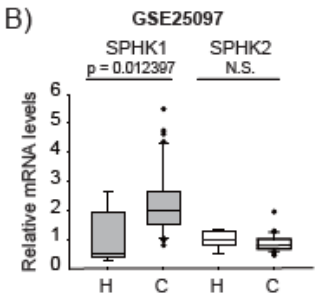
ANOVA and student Newman-Keuls tests were used to determined significant differences between groups and between the means of different groups. Results are expressed as mean ± standard error (SE).

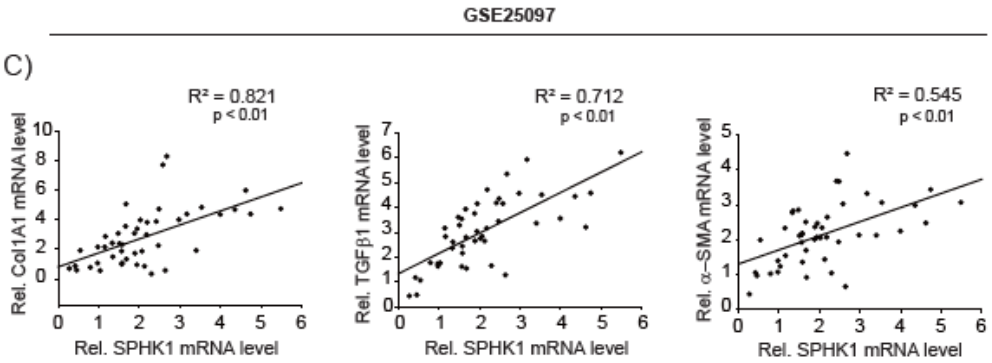
III. RESULTS

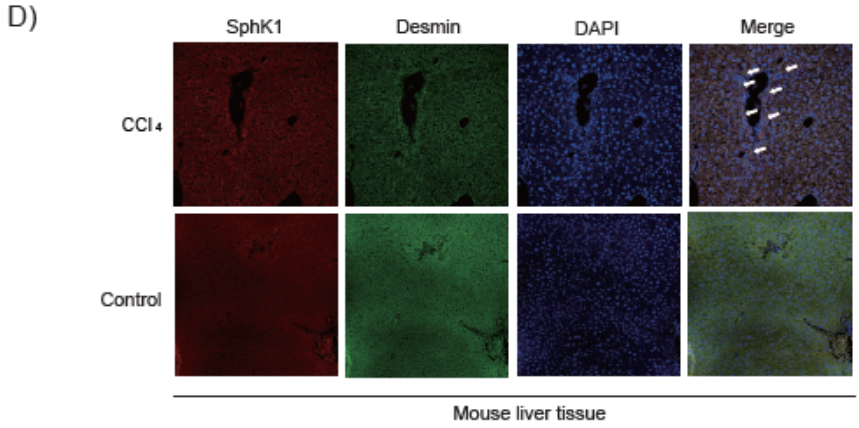
1. Overexpression of SphK1 in HSCs and fibrotic liver

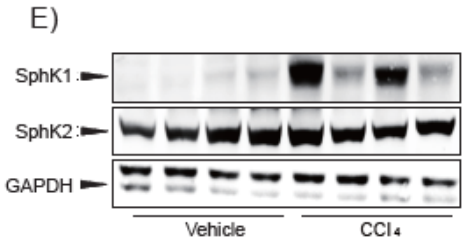
First, we examined the expression of SphK1 and desmin presenting HSC activation in the cirrhotic and adjacent normal liver tissues of patients with cancer to pursue the biological importance of SphK1 on liver fibrosis in clinical situation. As a result, it was found that the expressions of SphK1 and desmin were increased in samples from cirrhosis patients and were stained in the similar regions of samples, confirmative of SphK1 overexpression in activated HSCs (Figure 1A). Expression Omnibus database (GSE25097) showed that the transcript level of SphK1 was raised in patients possessing liver cirrhosis compared with those observed in healthy individuals (Figure 1B). However, levels of SphK2 were not changed in liver cirrhosis samples. Similarly, in the patients with liver cirrhosis, strong correlation existed between SphK1 and markers of liver fibrogenesis and HSC trans-differentiation (i.e. collagen 1A1, TGF-β, or α-SMA) suggestive of the role of SphK1 as a potential molecular marker of liver fibrosis (Figure 1C). Similar outcomes were detected in the livers of CCl_4-induced liver fibrosis animal model (Figure 1D). To intensify the connection among SphK1 and liver fibrosis, we investigated SphK1 expressions in liver homogenate of fibrotic animal samples. SphK1 levels, but not SphK2 were increased in liver homogenates from CCl_4-treated mice and BDL-induced mice than their respective controls (Figure 1E and F). Therefore, we consent to that SphK1 is upregulated in fibrotic liver conditions, which would progress liver fibrosis.











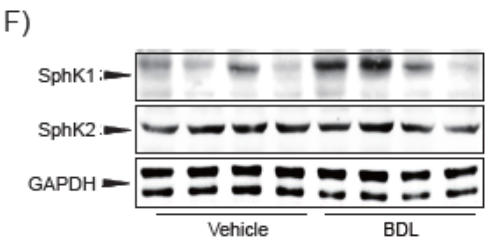


Figure 1. Overexpression of Sphingosine Kinase 1 (SphK1) in various fibrotic conditions

(A) Immunostaining of SphK1 and desmin in cirrhotic liver patients (magnification: 200x). (B) Transcript levels of SphK1 and SphK2 in patients with liver cirrhosis (C) or healthy individuals (N) from GSE25097 (n = 6-40). (C) Pearson's correlation analyses in a large cohort of fibrosis patients (GSE25097) (n=46). (D) Immunostaining of SphK1 and desmin in mice liver tissues treated with carbon tetrachloride (CCl_4). White arrows show colocalization of SphK1 and desmin (magnification: 200x). (E) Immunoblotting of SphK1in liver homogenates from CCl_4-treated mice liver tissue. GAPDH indicates equal loading of proteins. (F) Immunoblotting of SphK1 in liver homogenates from bile duct ligation (BDL) mice.

2. Induction of SphK1 during HSC activation

We analyzed the levels of SphK1 and SphK2 in primary hepatocytes and HSCs isolated from the livers of healthy mice, and found that SphK1 was more abundant in HSCs than hepatocytes (Figure 2A). The opposite changes occurred in case of SphK2. In addition, SphK1 was up-regulated in primary HSCs during culture activation along with α-SMA induction (Figure 2B). The transcript data from GSE34949 fortified the induction of SphK1 during HSC trans-differentiation (Figure 2C). Next, we examined SphK1 and SphK2 expression after stimulation with TGF-β, an essential profibrogenic cytokine in LX-2 cells (a human hepatic stellate cell line) from different time period. SphK1 was found to increase after 3-12 h of TGF-β treatment, which peaked at 6 h (Figure 2D). However, inductive effect of SphK2 by TGF-β was not observed. Consistently, TGF-β treatment enhanced the immunostaining levels of SphK1 (Figure 2E). When we utilized another hepatic stellate cell line from rats, HSC-T6 cells, we could reveal increased SphK1 expression derived from TGF-β, peaked at 24 h (Figure 2F). In addition, TGF-β-mediated SphK1 induction was significantly attenuated by SB431542, a TGF-β receptor antagonist, indicative of association of TGF-β signaling (Figure 2G). Our results demonstrate that SphK1 is up-regulated in activated HSCs during hepatic fibrogenesis.

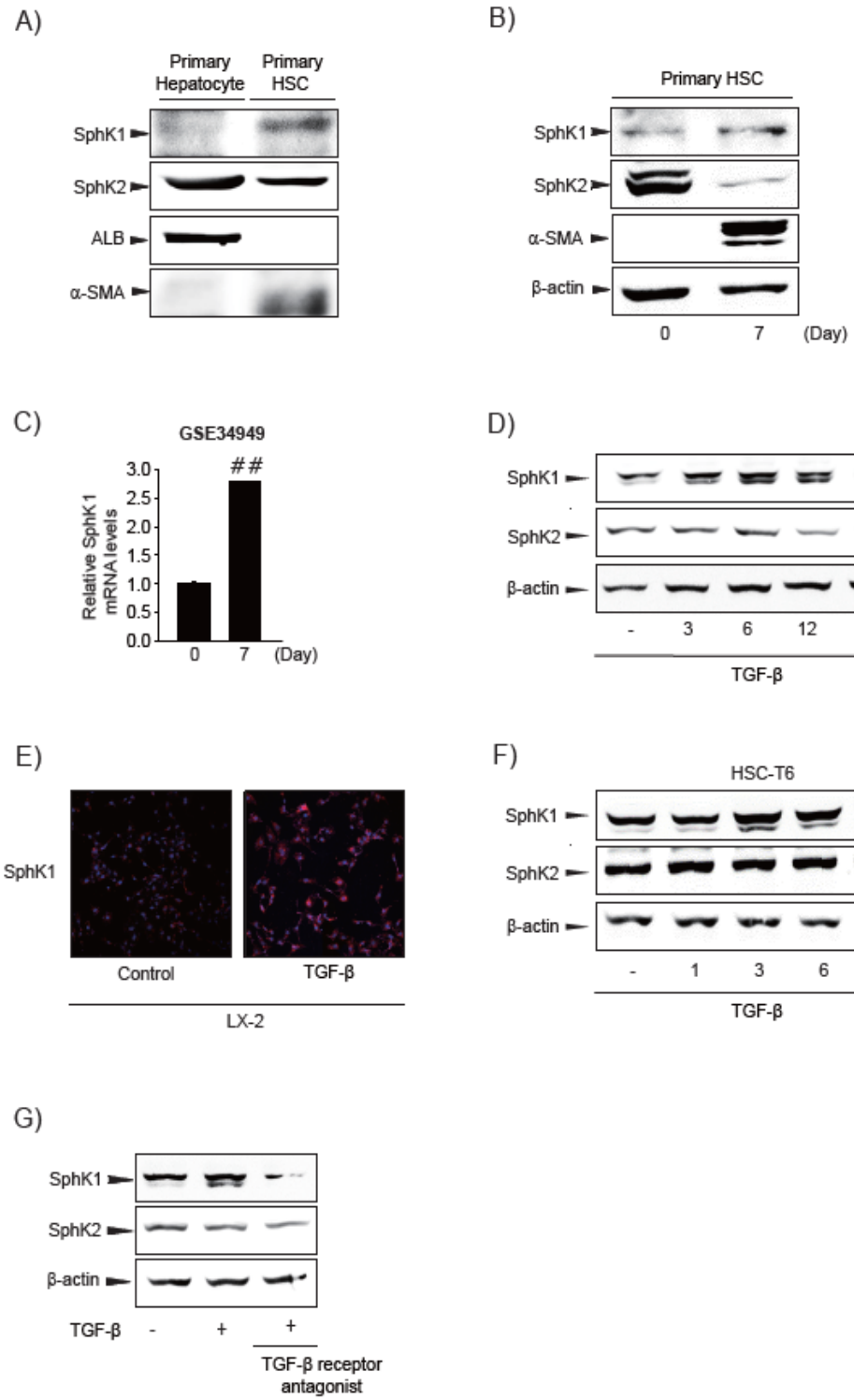


Figure 2. Upregulation of SphK1 during HSC activation

(A) Expression of SphK1/2 in mouse primary cell hepatocytes and quiescent HSCs. Immunoblotting was done on the cell lysates (20 μg each). α-smooth muscle actin (α-SMA) and albumin (ALB) were detected as marker of HSCs and hepatocytes, respectively. β-actin indicated equal loading of proteins. (B) Expression of SphK1/2 in quiescent or activated primary HSCs. Primary HSCs were isolated and cultured in growth medium for 0 (quiescent) or 7 (activated) days. (C) Transcript level of SphK1 in quiescent or activated primary HSCs from GSE34949. The data represents the mean ± SE (n = 3, significant different versus quiescent, $^{**}p<0.01$). (D) The effect of transforming growth factor-β (TGF-β) treatment on expression of SphK1/2. Immunoblotting analysis was performed with lysates of LX-2 cells treated with TGF-β (2 ng/mL) for 3-24 h. (E) Immunostaining of SphK1 in TGF-β-treated LX-2 Cells. (F) The effect of TGF-β on expression of SphK1/2 in HSC-T6. (G) The effect of TGF-β receptor antagonist on SphK1 induced by TGF-β in LX-2 cells. The cells were treated with TGF-β antagonist (10 μM) in the presence or absence of TGF-β. The level of SphK1 protein was analyzed after TGF-β (2 ng/mL) treatment for 6 h.

3. TGF-β-mediated SphK1 up-regulation by Smad3-dependent pathway

To delineate the regulatory mechanism controlling SphK1 induction, we scrutinized mRNA level of SphK1 after TGF-β treatment in LX-2 cells, and discovered that SphK1 transcript levels were steadily increased up to 6 h of treatment (Figure 3A). In case of SphK2, there was no change after TGF-β stimulation. Actinomycin-D (ActD), a transcriptional inhibitor, suppressed TGF-β-mediated SphK1 induction (Figure 3B). Thus, these data showed that SphK1 up-regulation by TGF-β was transcriptionally governed. We investigated whether Smad3, a well-known transcription factor for TGF-β signaling pathway which is the most critical cellular mechanistic pathway in hepatic fibrogenesis, is involved in this phenomenon. To test the role of Smad3 in the TGF-β-mediated SphK1 induction, we introduced Smad3 in LX-2 cells. We identified that TGF-β incubation of MOCK-transfected LX-2 cells enhanced SphK1 expression, which was further intensified by ectopic expression of Smad3 (Figure 3C). On the other hand, TGF-β-induced SphK1 up-regulation was substantially decreased by treatment with SIS3, a Smad3 inhibitor (Figure 3D). Our data showed that TGF-β-mediated SphK1 expression was occurred by Smad3-dependent pathway.

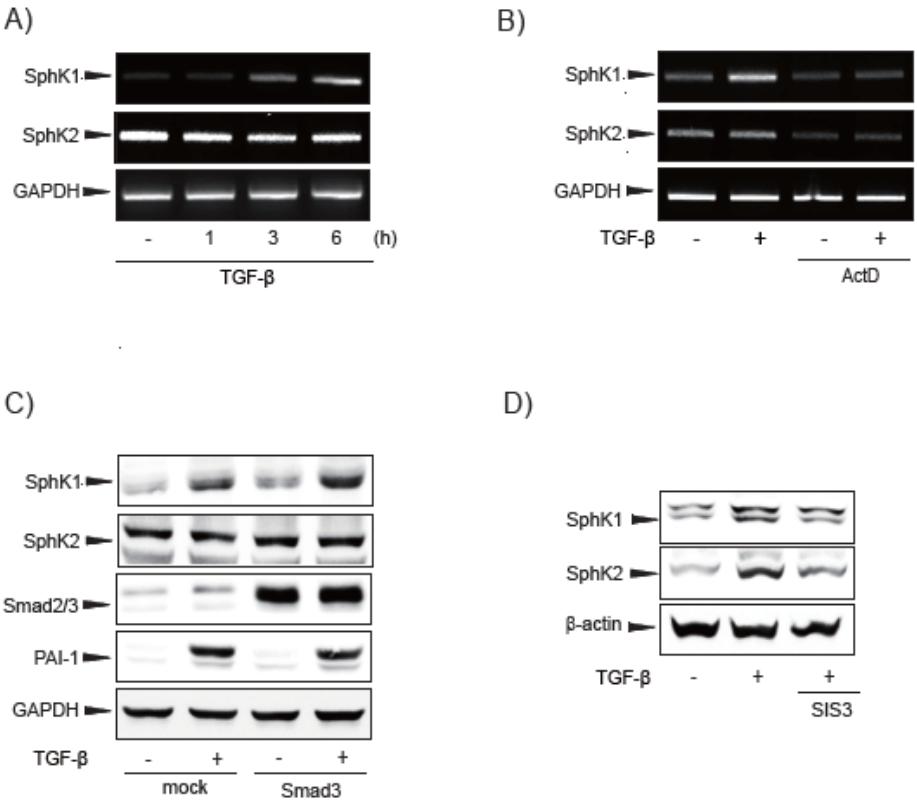


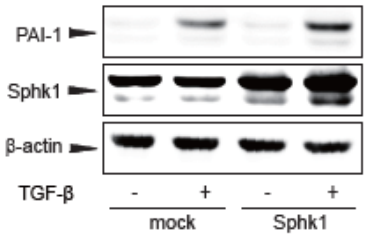
Figure 3. TGF-β-mediated SphK1 induction by Smad3-dependent pathway

(A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. LX-2 cells were treated with TGF-β (2 ng/mL) for 1-6 h. The mRNA expression of SphK1 and SphK2 using GAPDH as a control. (B) The potency of actinomycin-D (ActD) on SphK1 mRNA induction by TGF-β in LX-2 cells. The cells were treated with ActD (5 μg/mL) in the presence or absence of TGF-β (1 ng/mL). (C) The effect of Smad3 overexpression on TGF-β-induced SphK1 expression. LX-2 cells were transfected with Smad3 or pCDNA (MOCK), then treated with TGF-β (1 ng/mL) for 6 h. (D) The effect of Smad3 inhibitor (SIS3) on TGF-β-induced SphK1 expression. The cells were treated with SIS3 (5 μM) in the presence or absence of TGF-β (1 ng/mL). Immunoblotting was done to detect SphK1/2 levels.

4. The impact of SphK1 on liver fibrogenesis

To reveal whether SphK1 can exert on the liver fibrogenesis we checked the effect of SphK1 modulation on the expression of PAI-1, a fibrotic marker upon HSC activation. The PAI-1 level after treatment with TGF-β markedly increased by SphK1 (Figure 4A). However, knockdown of SphK1 by siRNA significantly attenuated TGF-β-induced PAI-1 expression as compared with control siRNA (Figure 4B). Similarly, incubation with SphK1 inhibitors (PF543, RB005) remarkably abolished PAI-1 induction by TGF-β (Figure 4C). These results supported that SphK1 was sufficient to promote liver fibrogenesis in HSCs.

A)



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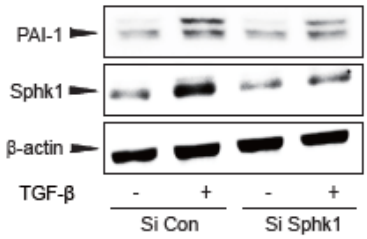

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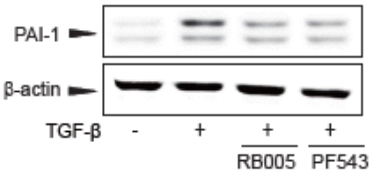
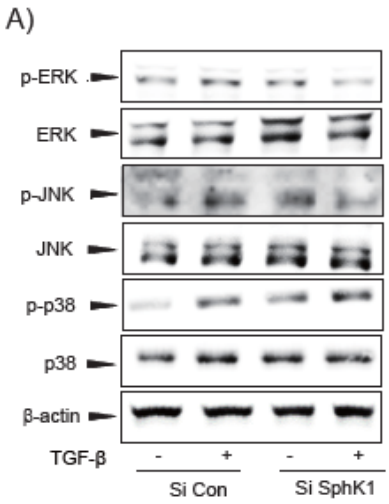


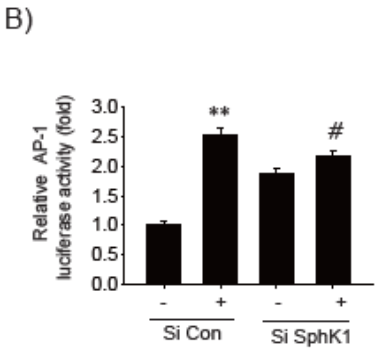
Figure 4. The effect of SphK1 on HSC activation and hepatic fibrogenesis.

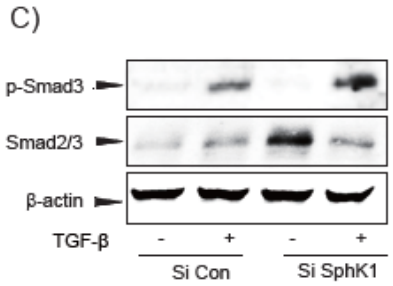
(A) The effect of SphK1 on TGF- β-mediated fibrogenic genes. LX-2 cells were transfected with SphK1 or pCDNA (MOCK) for 24 h and then cultivated with TGF- β (1 ng/ml) for 6 h. (B) The effect of SphK1 knockdown on TGF-β-induced fibrogenic genes. LX-2 cells were transfected with control siRNA (SiCon) or SphK1 siRNA (SiSphK1) for 24 h and then treated with TGF- β (1 ng/mL) for 6 h. (C) The effect of RB005 and PF543 on expression of TGF-β-induced fibrogenic genes. The cells were treated with PF543 (10 μM), RB005 (10 μM) in the presence or absence of TGF-β (1 ng/mL). Fibrogenic markers were detected by immunoblotting.

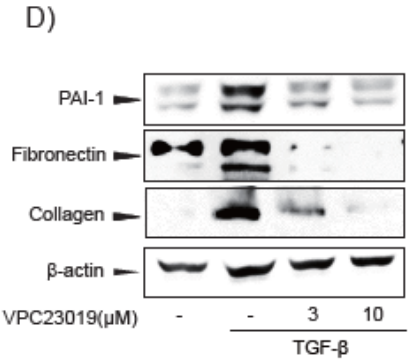
5. Involvement of MAPK in SphK1-caused liver fibrosis

Previously, it is proposed that AP-1-depedent regulatory roles for TGF-β signaling during liver fibrosis [21]. To investigate the mechanism by which SphK1 drives the fibrotic response, we sought to determine role of AP-1 in TGF-β-mediated liver fibrogenesis. The treatment of MOCK-transfected cells with TGF-β incremented MAPK phosphorylation such as ERK, JNK, and p38. However, knockdown of SphK1 decreased only increased ERK phosphorylation by TGF-β (Figure 5A). When we measured AP-1-dependent luciferase activity to evaluate the effect of SphK1 on TGF-β-induced AP-1 transactivation, we found that facilitated AP-1 luciferase activity by TGF-β was slightly suppressed by SphK1 knockdown (Figure 5B). Since Smad2/3 have been illustrated to act as another key transcription factor [22], we tested the possible link between Smad2/3 and SphK1-involved fibrogenic response. Unexpectedly, phosphorylation of Smad3 against TGF-β was not attenuated by introduction of SphK1 SiRNA (Figure 5C). Based on the fact that sphingosine-1-phosphate (S1P) is a bioactive lipid mediator formed by phosphorylation of sphingosine by SphK1 and activates HSCs via a S1P receptor (S1PR) 1 or S1PR3-dependent manner [23], we assessed the possibility of SphK1-mediated liver fibrogenesis via S1PRs. S1PR1/3 inhibition using a chemical inhibitor (VPC23019) exerted anti-fibrogenic effects against TGF-β which was associated with MAPK activation (Figure 5D and 5E). These results provided evidence that SphK1overexpression in HSCs might facilitate MAPK activation to promote hepatic fibrogenesis, and this event could be caused by S1PRs.









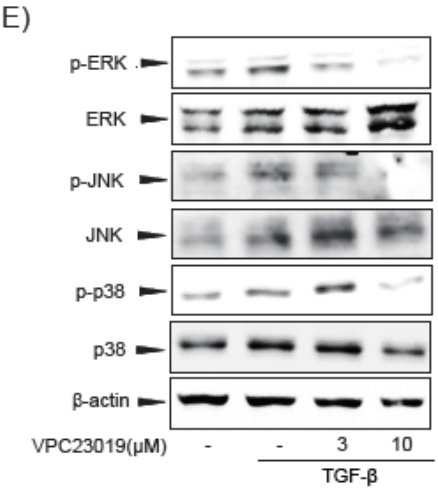
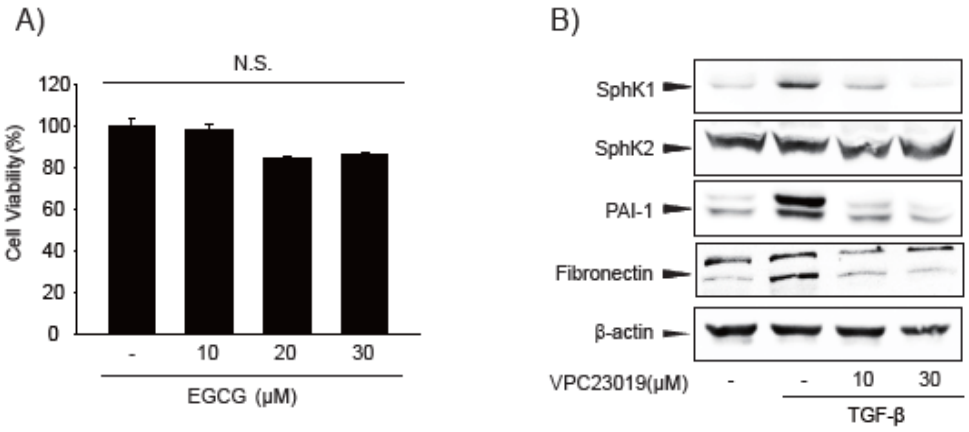


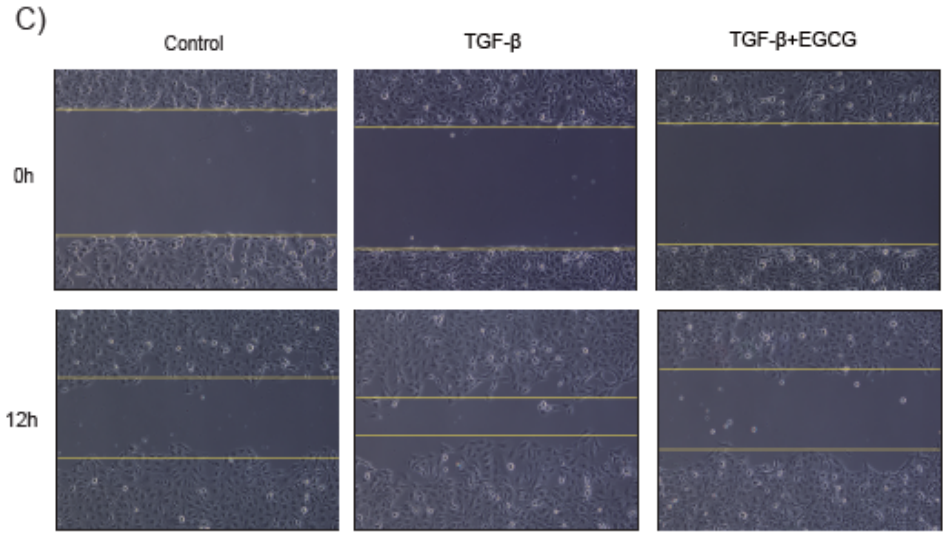
Figure 5. Involvement of AP-1 in SphK1-mediated hepatic fibrogenesis.

(A) The effect of SphK1 knockdown on TGF-β-induced MAPK activation. LX-2 cells were transfected with control siRNA (SiCon) or SphK1 siRNA (SiSphK1) for 24 h and then treated with TGF-β (1 ng/mL) for 3 min. (B) The effect of SphK1 knockdown on AP-1 luciferase activity. LX-2 cells were transiently co-transfected with AP-1 luciferase plasmids and control siRNA (SiCon) or SphK1 siRNA (SiSphK1) for 24 h and then treated with TGF-β (1 ng/mL) for 12 h. Data represent means ± S.E. of three replicates; significant as compared with vehicle-treated cells, $^{**}p<0.01$, significant as compared with TGF-β-treated cells, $^{\#}p<0.05$. (C) The effect of SphK1 knockdown on TGF-β-induced Smad phosphorylation. Cells were transfected with control siRNA (siCon) or SphK1 siRNA (siSphK1) for 24 h and then treated with TGF-β (1 ng/mL) for 30 min. (D) The effect of sphingosine-1-phosphate receptor 1/3 inhibitor (VPC23019) on TGF-β-mediated fibrogenic gene induction. The cells were treated with VPC23019 (3, 10 μM) in the presence or absence of TGF-β (1 ng/mL). (E) The effect of VPC23019 on TGF-β-mediated MAPK activity. The cells were treated with VPC23019 (3, 10 μM) in the presence or absence of TGF-β (1 ng/mL) for 3 min.

6. Improvement of SphK1-associated liver fibrogenesis by EGCG

It has been demonstrated that EGCG, the most abundant polyphenolic compounds, has a protective role in liver fibrosis [24]. Unfortunately, its mechanism of action on attenuation of liver fibrosis is still necessary. Hence, we sought to determine whether EGCG diminished SphK1-dependent liver fibrogenic event. When we evaluated the cytotoxicity of EGCG in LX-2 cells, cell viability was no significant differences between vehicle and EGCG- treated groups (Figure 6A). We explored whether EGCG modulated SphK1 expression in HSCs. Interestingly, EGCG reduced SphK1 levels in a dose dependent manner without change of SphK2 levels (Figure 6B). At the same time, we observed EGCG suppressed expression of fibrogenic genes. The existence of SphK1within fibrotic septa of human cirrhosis liver tissues implicated that it might contribute to remodeling of the scar via cell migration. This prompted us to examine the effect of TGF-β-mediated SphK1 on wound healing. The wound that were cultivated with TGF-β markedly narrower than controls. In contrast, cells migration into the wound area was dramatically diminished after EGCG incubation (Figure 6C). In addition, EGCG treatment attenuated TGF-β-mediated MAPK phosphorylation and AP-1 luciferase activity (Figure 6D and 6E). Intriguingly, EGCG had the inhibitory effects on Smad3 phosphorylation and SBE luciferase activity (Figure 6F and 6G). Therefore, our data corroborated the antifibrotic effect of EGCG on SphK1-derived liver fibrosis and consequent liver fibrosis.





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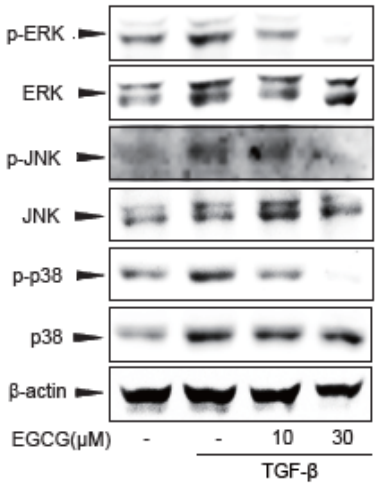

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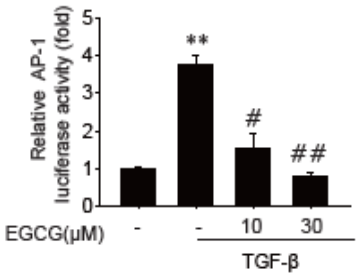

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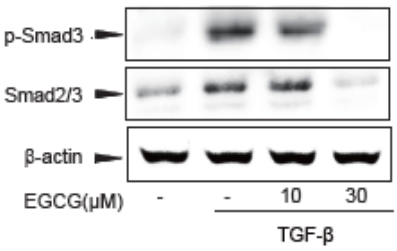

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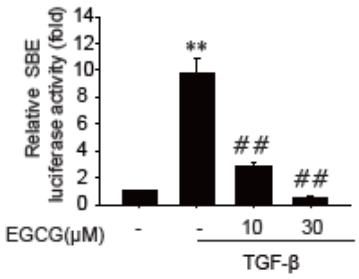

Figure 6. Suppressive effect of epigallocatechin-3-gallate (EGCG) on SphK1-dependent hepatic fibrogenesis.

(A) Cytotoxicity evaluation of EGCG. LX-2 cells were treated with EGCG for 12 h, and then cell viability was assessed employ a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Data represent means ± S.E. of three replicates. (B) The effect of EGCG on expression of TGF-β-induced SphK1/2 and fibrogenic genes. The cells were treated with EGCG (10, 30 μM) in the presence or absence of TGF-β (1 ng/mL). SphK1/2 and fibrogenic markers were finded by immunoblotting. (C) The effect of EGCG on TGF-β-induced cell migration by wound healing assay. LX-2 cells were treated with EGCG (10, 30 μM) in the presence or absence of TGF-β (1 ng/mL) after creating wounds. (D) The effect of EGCG on TGF-β-induced MAPK transactivation. The cells were treated with EGCG (10, 30 μM) in the presence or absence of TGF-β (1 ng/mL) for 3 min. (E) The effect of EGCG on AP-1luciferase activity. LX-2 cells were temporarily transfected with AP-1 plasmids for 24 h and then treated with TGF-β (1 ng/mL) for 12 h. Data represent means ± S.E. of three replicates; significant as compared with vehicle-treated cells, $^{**}p<0.01$, significant as compared with TGF-β-treated cells, $^{\#\#}p<0.01$ (F) The effect of EGCG inhibition on TGF-β-induced Smad phosphorylation. The cells were treated with EGCG (10, 30 μM) in the presence or absence of TGF-β (1 ng/mL) for 30 min. (G) The effect of EGCG on SBE luciferase activity. LX-2 cells were transfected with SBE luciferase plasmid for 24 h and then cultivated with TGF-β (1 ng/mL) for 12 h. Data represent means ± S.E. of three replicates; significant as compared with vehicle-treated cells, $^{**}p<0.01$, significant as compared with TGF-β-treated cells, $^{\#\#}p<0.01$

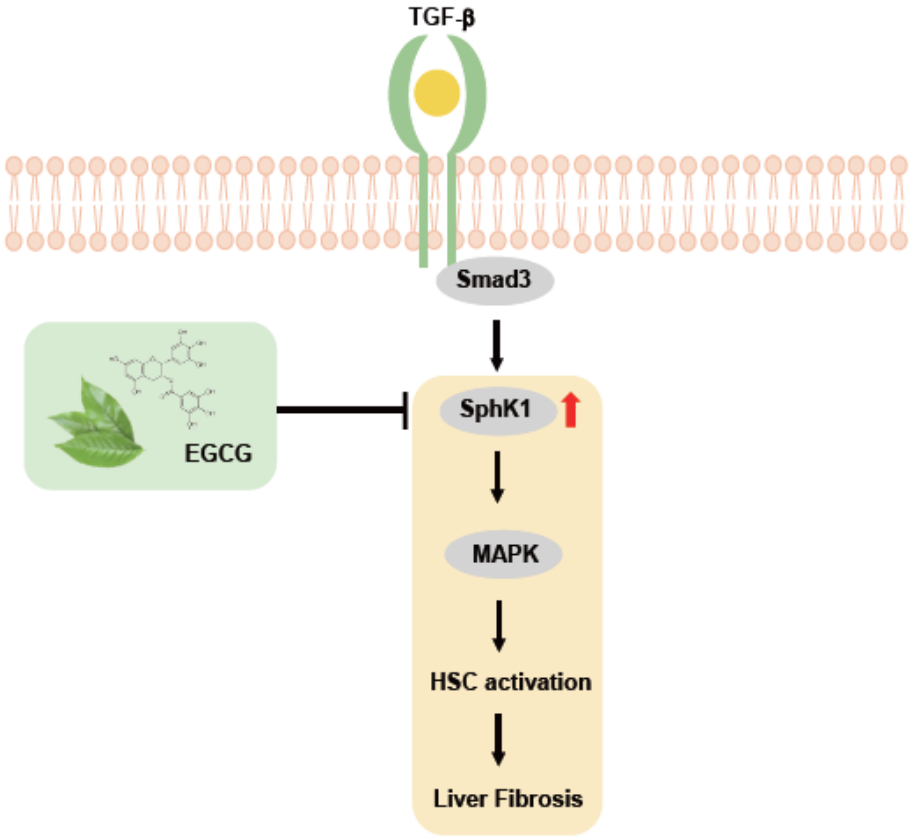

Figure 7.

Schematic diagram representing role of SphK1 on hepatic stellate cell activation and its antagonistic effect of EGCG.

IV. DISCUSSION

Persistent HSC phenotypic changes upon activation is derived from several environmental stimuli [11, 12, 18]. TGF-β has been considered as a pivotal cytokine for fibrogenic events accompanying accumulation of matrix protein such as collagen and fibronectin [11]. S1P, another crucial liver fibrosis mediator, is produced by SphK1 which is widely distributed and catalyzes the conversion from sphingosine to S1P [17, 25]. The fact that the liver is a major organ for regulating SphK levels [26] suggests the role of SphK1 on S1P regulation in liver pathophysiology. Despite the known effect of SphK1/S1P/S1PRs signaling axis on liver fibrosis [18], the molecular basis for SphK1 in the progression of liver fibrosis is not revealed. In the present study, we discovered that SphK1 was overexpressed in HSCs under fibrotic conditions of liver. GEO data illustrating that elevated SphK1 expression in patient sample with cirrhotic liver and positive correlation between SphK1 and fibrosis-related genes support the regulatory role of SphK1 in HSC transdifferentiation and liver fibrosis (Figure 1A and B). This was strengthened by data from two different fibrosis mice model (Figure 1E and F). SphK1 upregulation in primary HSCs during activation and TGF-β-treated LX-2 cells also fortified our findings (Figure 2B and 2D). In addition, we implicated the significance of SphK1 in HSC biology based on the observation that abundancy of SphK1 in HSCs than hepatocytes. Furthermore, we observed that SphK2 was not comparably enhanced in activated primary HSCs and TGF-β-treated LX-2 cells. Although SphK2 is more abundant in liver than SphK1 [27], our result indicated the greater role for SphK1 in HSC transdifferentiation.

It has been considered that the SphK1/S1P pathway is critically involved in the mechanism by which TGF-β-elicited profibrotic effect in diverse types of fibroblasts [28-30]. In this regard, SphK1 overexpression has been explained to be important for TGF-β-related signaling pathway. This notion was assisted by our results of SphK1 level after treatment with TGF-β receptor antagonist in LX-2 cells (Figure 2F). Moreover, we found that Smad3, the most

crucial transcription factor for TGF-β signaling [11], as an upstream regulator of SphK1 (Figure 3C), which was consistently reported in fibroblast [31]. Besides, transcriptional mediations such as specificity protein 1, hypoxia-induced factors, or AP-1, and several microRNA (miR) (i.e. miR-124, miR-506, miR-125b, or miR-613) also exerted expression of SphK1 [32-37]. The detailed mechanistic study in the association between Smad3 and SphK1 has to be elucidated in the further study. Moreover, we found that Rho/ROCK signaling, another pathway activated by TGF-β [38], was related to modulation of SphK1 expression in our experiment (data not shown). Furthermore, we tried to regulatory mechanism for SphK1 activity, which was influenced by several kinase such as ERK and Fyn [39, 40]. Unfortunately, we could not observe that TGF-β phosphorylated SphK1 in LX-2 cells. Hence, it is still needed to identify the mechanistic study for regulation of SphK1 activity besides its expression.

TGF-β-transmitting intracellular signaling in liver fibrosis is mainly mediated by Smad2/3, but it is also accepted that additional TGF-β responses is related to the activation of Smad2/3-independent pathways such as MAPK [8, 11]. Our data indicated that intensification of TGF-β-related HSC activation by SphK1 relied on activation of MAPK, especially ERK. This outcome is reinforced by the fact that ERK is a main downstream of SphK1/S1P pathway [41]. Unexpectedly, SphK1 did not affect the activation of canonical Smad signaling pathway by TGF-β treatment. Evidence that treatment with S1PR1/3 antagonist attenuated TGF-β-derived hepatic fibrogenic response via MAPK activation implicates the SphK1/S1P/S1PRs axis. Despite our finding on ERK as a direct mediator of SphK1-mediated HSC activation, S1PR1/3 antagonist challenge changed all three MAPK activities of ERK, p38, and JNK.

As an effort to develop an efficient therapeutic agent for liver fibrosis, we adopted EGCG, one of the most abundant polyphenols present green tea [42], and investigated the effects on SphK1-derived liver fibrosis. It has been recently reported that EGCG can control various signal transduction in HSCs in connection with PDGF, glutathione synthesis, collagen production, and collagenase activity [24, 43-45]. In our study, we found the novel effect of

EGCG on SphK expression. EGCG selectively diminished SphK1 expression, but not SphK2 level, indicative of great role of EGCG on HSC activation and subsequent liver fibrosis as SphK1. In addition, EGCG had an antifibrotic effect presenting by attenuation of fibrogenic genes and wound healing. This significant antifibrotic effect of EGCG was mediated by both MAPK and Smad-signaling pathway as in previous studies [46]. Discrepancy of data on related signaling between SphK1 modulation and EGCG treatment may due to multiple actions of EGCG would have and/or indirect effects on liver fibrosis. Further *in vivo* studies utilizing EGCG are necessary to strengthen our hypothesis, and these are currently being pursued in our laboratory.

In conclusion, our study demonstrates that SphK1 overexpression elicited by Smad3, which activates HSCs by MAPK activation, and this event was suppressed by EGCG (Figure 7). These outcomes suggest new insight into the regulation of a prime signaling pathway, which attenuates hepatic fibrogenesis and consequent liver fibrosis.

V. REFERENCES

1. Seki, E. and D.A. Brenner, *Recent advancement of molecular mechanisms of liver fi brosis.* J Hepatobiliary Pancreat Sci, 2015. **22**(7): p. 512-8.
2. Verrecchia, F. and A. Mauviel, *Transforming growth factor-beta and fibrosis.* World J Gastroenterol, 2007. **13**(22): p. 3056-62.
3. Gao, C., et al., *Transforming growth factor beta (TGF-beta) expression in isolated and cultured rat hepatocytes.* J Cell Physiol, 1996. **167**(3): p. 394-405.
4. Friedman, S.L., *Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver.* Physiol Rev, 2008. **88**(1): p. 125-72.
5. Friedman, S.L., *Cytokines and fibrogenesis.* Semin Liver Dis, 1999. **19**(2): p. 129-4 0.
6. Massague, J., *TGFbeta signalling in context.* Nat Rev Mol Cell Biol, 2012. **13**(10): p. 616-30.
7. Zhang, Y.E., *Non-Smad pathways in TGF-beta signaling.* Cell Res, 2009. **19**(1): p. 128-39.
8. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in T GF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-84.
9. Gressner, A.M., et al., *Roles of TGF-beta in hepatic fibrosis.* Front Biosci, 2002. **7**: p. d793-807.
10. Kutz, S.M., et al., *TGF-beta1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion.* J Cell Sci, 2001. **114**(Pt 21): p. 3905-14.
11. Breitkopf, K., et al., *TGF-beta/Smad signaling in the injured liver.* Z Gastroenterol, 2006. **44**(1): p. 57-66.
12. Breitkopf, K., et al., *Expression patterns of PDGF-A, -B, -C and -D and the PDG F-receptors alpha and beta in activated rat hepatic stellate cells (HSC).* Cytokine, 2005. **31**(5): p. 349-57.
13. Vinas, O., et al., *Human hepatic stellate cells show features of antigen-presenting c ells and stimulate lymphocyte proliferation.* Hepatology, 2003. **38**(4): p. 919-29.
14. Pyne, N.J., et al., *Sphingosine 1-phosphate and cancer.* Adv Biol Regul, 2018. **68**: p. 97-106.
15. Orr Gandy, K.A. and L.M. Obeid, *Targeting the sphingosine kinase/sphingosine 1-ph osphate pathway in disease: review of sphingosine kinase inhibitors.* Biochim Bioph ys Acta, 2013. **1831**(1): p. 157-66.
16. Maceyka, M., et al., *Sphingosine-1-phosphate signaling and its role in disease.* Tren ds Cell Biol, 2012. **22**(1): p. 50-60.
17. Spiegel, S. and S. Milstien, *Sphingosine-1-phosphate: an enigmatic signalling lipid.* Nat Rev Mol Cell Biol, 2003. **4**(5): p. 397-407.
18. Li, C., et al., *Sphingosine 1-phosphate (S1P)/S1P receptors are involved in human l iver fibrosis by action on hepatic myofibroblasts motility.* J Hepatol, 2011. **54**(6): p. 1205-13.
19. Kim, K.M., et al., *Galpha(12) overexpression induced by miR-16 dysregulation contr ibutes to liver fibrosis by promoting autophagy in hepatic stellate cells.* J Hepatol, 2018. **68**(3): p. 493-504.
20. Yang, J.H., et al., *Inhibitory Effect of Sestrin 2 on Hepatic Stellate Cell Activation*

and Liver Fibrosis. Antioxid Redox Signal, 2019. **31**(3): p. 243-259.
21. Cho, S.S., et al., *REDD1 attenuates hepatic stellate cell activation and liver fibrosis via inhibiting of TGF-beta/Smad signaling pathway.* Free Radic Biol Med, 2021. **176**: p. 246-256.
22. Massague, J., J. Seoane, and D. Wotton, *Smad transcription factors.* Genes Dev, 2005. **19**(23): p. 2783-810.
23. Liu, X., et al., *Essential roles of sphingosine 1-phosphate receptor types 1 and 3 in human hepatic stellate cells motility and activation.* J Cell Physiol, 2011. **226**(9): p. 2370-7.
24. Sakata, R., et al., *Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90.* J Hepatol, 2004. **40**(1): p. 52-9.
25. Yang, L., et al., *Sphingosine kinase/sphingosine 1-phosphate (S1P)/S1P receptor axis is involved in liver fibrosis-associated angiogenesis.* J Hepatol, 2013. **59**(1): p. 114-23.
26. Pappu, R., et al., *Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate.* Science, 2007. **316**(5822): p. 295-8.
27. Kihara, A., Y. Anada, and Y. Igarashi, *Mouse sphingosine kinase isoforms SPHK1a and SPHK1b differ in enzymatic traits including stability, localization, modification, and oligomerization.* J Biol Chem, 2006. **281**(7): p. 4532-9.
28. Kono, Y., et al., *Sphingosine kinase 1 regulates differentiation of human and mouse lung fibroblasts mediated by TGF-beta1.* Am J Respir Cell Mol Biol, 2007. **37**(4): p. 395-404.
29. Gellings Lowe, N., et al., *Sphingosine-1-phosphate and sphingosine kinase are critical for transforming growth factor-beta-stimulated collagen production by cardiac fibroblasts.* Cardiovasc Res, 2009. **82**(2): p. 303-12.
30. Yamanaka, M., et al., *Sphingosine kinase 1 (SPHK1) is induced by transforming growth factor-beta and mediates TIMP-1 up-regulation.* J Biol Chem, 2004. **279**(52): p. 53994-4001.
31. Cencetti, F., et al., *Transforming growth factor-beta1 induces transdifferentiation of myoblasts into myofibroblasts via up-regulation of sphingosine kinase-1/S1P3 axis.* Mol Biol Cell, 2010. **21**(6): p. 1111-24.
32. Sobue, S., et al., *Transcription factor specificity protein 1 (Sp1) is the main regulator of nerve growth factor-induced sphingosine kinase 1 gene expression of the rat pheochromocytoma cell line, PC12.* J Neurochem, 2005. **95**(4): p. 940-9.
33. Cuvillier, O. and I. Ader, *Hypoxia-inducible factors and sphingosine 1-phosphate signaling.* Anticancer Agents Med Chem, 2011. **11**(9): p. 854-62.
34. Xia, J., et al., *miR-124 inhibits cell proliferation in gastric cancer through down-regulation of SPHK1.* J Pathol, 2012. **227**(4): p. 470-80.
35. Wang, D., et al., *MicroRNA-506-3p initiates mesenchymal-to-epithelial transition and suppresses autophagy in osteosarcoma cells by directly targeting SPHK1.* Biosci Biotechnol Biochem, 2019. **83**(5): p. 836-844.
36. Zhao, X., et al., *MiRNA-125b inhibits proliferation and migration by targeting SphK1 in bladder cancer.* Am J Transl Res, 2015. **7**(11): p. 2346-54.
37. Yu, H., et al., *miR-613 inhibits bladder cancer proliferation and migration through targeting SphK1.* Am J Transl Res, 2017. **9**(3): p. 1213-1221.
38. Ji, H., et al., *Rho/Rock cross-talks with transforming growth factor-beta/Smad pathway participates in lung fibroblast-myofibroblast differentiation.* Biomed Rep, 2014. **2**(6): p. 787-792.
39. Pitson, S.M., et al., *Activation of sphingosine kinase 1 by ERK1/2-mediated phospho*

rylation. EMBO J, 2003. **22**(20): p. 5491-500.

40. Olivera, A., et al., *IgE-dependent activation of sphingosine kinases 1 and 2 and sec retion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses.* J Biol Chem, 2006. **281**(5): p. 2515-25.
41. Kim, J.H., et al., *Sphingosine 1-phosphate activates Erk-1/-2 by transactivating epid ermal growth factor receptor in rat-2 cells.* IUBMB Life, 2000. **50**(2): p. 119-24.
42. Yu, D.K., et al., *The anti-fibrotic effects of epigallocatechin-3-gallate in bile duct-lig ated cholestatic rats and human hepatic stellate LX-2 cells are mediated by the PI3 K/Akt/Smad pathway.* Acta Pharmacol Sin, 2015. **36**(4): p. 473-82.
43. Nakamuta, M., et al., *Epigallocatechin-3-gallate, a polyphenol component of green t ea, suppresses both collagen production and collagenase activity in hepatic stellate cells.* Int J Mol Med, 2005. **16**(4): p. 677-81.
44. Yumei, F., et al., *The antifibrogenic effect of (-)-epigallocatechin gallate results from the induction of de novo synthesis of glutathione in passaged rat hepatic stellate c ells.* Lab Invest, 2006. **86**(7): p. 697-709.
45. Zhen, M.C., et al., *Green tea polyphenol epigallocatechin-3-gallate suppresses rat h epatic stellate cell invasion by inhibition of MMP-2 expression and its activation.* A cta Pharmacol Sin, 2006. **27**(12): p. 1600-7.
46. Tipoe, G.L., et al., *Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, ox idative stress and fibrosis in carbon tetrachloride (CCl4)-induced liver injury in mic e.* Toxicology, 2010. **273**(1-3): p. 45-52.