

S1P 3-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species

メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/21766

S1P₃-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species

Noriko Takuwa^{1, 10}, Sei-Ichiro Ohkura^{1,2}, Shin-Ichiro Takashima^{1,2}, Keisuke Ohtani², Yasuo Okamoto¹, Tamotsu Tanaka³, Kaoru Hirano³, Soichiro Usui², Fei Wang¹, Wa Du¹, Kazuaki Yoshioka¹, Yoshiko Banno⁴, Motoko Sasaki⁶, Ikuyo Ichi⁷, Miwa Okamura⁵, Naotoshi Sugimoto¹, Kiyomi Mizugishi¹, Yasuni Nakanuma⁶, Isao Ishii⁹, Masayuki Takamura², Shuichi Kaneko², Shosuke Kojo⁷, Kiyoshi Satouchi³, Kunitoshi Mitumori⁵, Jerold Chun⁸, and Yoh Takuwa¹

Departments of ¹Physiology, ²Disease Control and Homeostasis, and ⁶Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Ishikawa 920-8640, ³Department of Applied Biological Science, Fukuyama University, Fukuyama, Hiroshima 729-0292, ⁴Department of Cell Signaling, Gifu University Graduate School of Medicine, Gifu 501-1194, ⁵Department of Veterinary Medicine, Tokyo University of Agricultural Technology, Fuchu 183-8509, Tokyo, ⁷Department of Food Science and Nutrition, Nara Women's University, Nara 630-8506, ⁸Department of Molecular Biology, The Helen L. Dorris Child and Adolescent Neuropsychiatric Disorder Institute, The Scripps Research Institute, La Jolla, CA 92037 USA, ⁹Department of Molecular and

Cellular Neurobiology, Gunma University Graduate School of Medicine, Maebashi,
Gunma 371-8511 and ¹⁰Department of Health and Medical Sciences, Ishikawa
Prefectural Nursing University, Kahoku, Ishikawa 929-1212, Japan

Running title: Sphingosine kinase, cardiac fibrosis and I/R injury

5395 words

Address correspondence to: Yoh Takuwa, M.D., Ph.D.

Department of Physiology, Kanazawa University Graduate School of Medicine

13-1 Takara-machi, Kanazawa

Ishikawa 920-8640, Japan

FAX:+81-76-234-4223

TEL:+81-76-265-2165

E-Mail: ytakuwa@med.kanazawa-u.ac.jp

Key words: sphingosine kinase-1 transgenic mouse, cardiac fibrosis,
ischemia/reperfusion injury, S1P₃, reactive oxygen species

Abstract

S1P₃-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species

Noriko Takuwa, Sei-Ichiro Ohkura, Shin-Ichiro Takashima, Keisuke Ohtani, Yasuo Okamoto, Tamotsu Tanaka, Kaoru Hirano, Soichiro Usui, Fei Wang, Wa Du, Kazuaki Yoshioka, Yoshiko Banno, Motoko Sasaki, Ikuyo Ichi, Miwa Okamura, Naotoshi Sugimoto, Kiyomi Mizugishi, Yasuni Nakanuma, Isao Ishii, Masayuki Takamura, Shuichi Kaneko, Shosuke Kojo, Kiyoshi Satouchi, Kunitoshi Mitumori, Jerold Chun, and Yoh Takuwa

Aim: Sphingosine kinase 1 (SPHK1), its product sphingosine-1-phosphate (S1P) and S1P receptor subtypes have been suggested to play protective roles for cardiomyocytes in animal models of ischemic preconditioning and cardiac ischemia/reperfusion injury. To get more insight into roles for SPHK1 *in vivo*, we have generated SPHK1-transgenic (TG) mice and analyzed cardiac phenotype.

Methods and Results: SPHK1-TG mice overexpressed SPHK1 in diverse tissues, with a nearly twenty-fold increase in enzymatic activity. The TG mice grew normally with normal blood chemistries, cell counts, heart rate and blood pressure. Unexpectedly, TG mice with high but not low expression levels of SPHK1 developed progressive myocardial degeneration and fibrosis, with upregulation of embryonic genes, elevated RhoA and Rac1 activity, stimulation of Smad3 phosphorylation and increased levels of oxidative stress markers. Treatment of juvenile TG mice with pitavastatin, an established inhibitor of the Rho family G proteins, or deletion of S1P₃, a major myocardial S1P receptor subtype that couples to Rho GTPases and transactivates Smad signaling, both inhibited cardiac fibrosis with concomitant inhibition of SPHK1-dependent Smad-3 phosphorylation. In addition, the anti-oxidant *N*-2-mercapto-propionylglycine, that reduces reactive oxygen species (ROS), also inhibited cardiac fibrosis. In *in vivo* ischemia/reperfusion injury, the size of myocardial infarct was 30% decreased in SPHK1-TG mice compared with wild-type mice.

Conclusion: These results suggest that chronic activation of the SPHK1-S1P signaling results in both the pathological cardiac remodeling through ROS mediated by S1P₃ and favorable cardio-protective effect.

1. Introduction

Sphingosine kinases (SPHKs) catalyze phosphorylation of sphingosine to produce pleiotropic lysophospholipid mediator sphingosine-1-phosphate (S1P), which has attracted much attention because of its diverse effects in a variety of cell types.^{1,2} These include stimulation of cell proliferation, inhibition of apoptosis and regulation of cell shape and cell motility, among others. SPHK1 and SPHK2, the two isozymes thus far identified, are ubiquitously but differentially expressed during embryogenesis and in adult tissues.¹ Previous studies^{3,4} have demonstrated that either SPHK1 or SPHK2 single knockout (KO) mice are phenotypically normal, whereas SPHK1/SPHK2 double KO mice are embryonic lethal with undetectable S1P levels.⁵ These observations indicate that S1P is required for mammalian embryogenesis, and is produced exclusively by SPHK1 and SPHK2 *in vivo* at least during embryonic period.

Most of the S1P effects are mediated through members of the G protein-coupled S1P receptor family, which include ubiquitously expressed subtypes, S1P₁, S1P₂ and S1P₃.^{6,7} The receptor repertoire of heterotrimeric G protein coupling to downstream signaling pathways, including the Rho family small GTPases, is responsible for diverse S1P actions.^{6,7} For example, S1P₁ mediates S1P-directed cell migration and other biological effects through activation of the Rho family small GTPase Rac1 via G_i, whereas S1P₂

mediates inhibition of cell migration through activation of Rho and resultant Rac inhibition via $G_{12/13}$.⁶ S1P₃ couples to G_i and $G_{12/13}$, mediating activation of both Rac and Rho, as well as activation of phospholipase C via G_q .⁶ In the cardiovascular system S1P₃ is abundantly expressed on cardiomyocytes, cardiac fibroblasts, vascular endothelial and smooth muscle cells, whereas S1P₁ and S1P₂ are mainly expressed on vascular endothelial and smooth muscle cells, respectively.⁶⁻¹² S1P₃ KO mice are phenotypically normal,¹³ however, S1P₃ deletion abrogates negative chronotropic and hypertensive effects after intravenous administration of S1P *in vivo*.¹⁴ Recent investigations in the cardiovascular system have suggested the involvement of the SPHK1-S1P signaling system in the protection of myocardium from ischemia/reperfusion (I/R) injury. The protective effect of S1P from I/R injury was abrogated by deletion of S1P₃.¹⁵ Deletion of both S1P₂ and S1P₃, but not either alone, resulted in aggravation of myocardial infarction due to ischemia/reperfusion (IR) injury.¹⁶ Deletion of SPHK1 sensitized the myocardium to I/R injury.¹⁷ However, the effects of chronic activation of the SPHK1-S1P signaling system in the heart *in vivo* have not yet been addressed thus far.

In order to evaluate the effects of endogenous overproduction of S1P on the cardiovascular system *in vivo*, we generated SPHK1-transgenic (TG) mice that

overexpress functional SPHK1 isoform in diverse tissues with more than 10-fold increases in enzymatic activity. We found that SPHK1a TG mice showed 100% occurrence of cardiac fibrosis, which is associated with cardiomyocyte degeneration, but without cardiac hypertrophy or hypertension. The development of cardiac fibrosis mechanistically involved activation of the S1P₃-Rho family small G protein signaling pathway and increased reactive oxygen species (ROS). SPHK1a TG mice also exhibited attenuation of myocardial infarct due to I/R injury compared with wild-type (WT) mice, indicating that SPHK1a overexpression has both the favorable and unfavorable effects on the heart.

2. Methods

2.1. Generation of SPHK1 TG Mice and S1P₃ knock-out (KO)/SPHK1 TG Bigenic Mice

We generated SPHK1 TG mice that overexpress SPHK1a in a variety of tissues under the universal CAG promoter (see Supplementary Materials for details). S1P₃ knock-out (KO) mice in C57BL/6 genetic background¹³ were mated with SPHK1 TG mice. All experiments using mice were approved by and performed according to the Guidelines for the care and Use of laboratory Animals in Kanazawa University, which strictly

conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Measurements of SPHK Activity and Levels of S1P, Dihydrosphingosine-1-phosphate (DH-S1P), Sphingosine, and Ceramide

SPHK activity in homogenates of the heart and other tissues was measured by using sphingosine and [γ - 32 P]ATP as substrates.⁵ Labeled lipids were analyzed as described⁸. The contents of S1P, DH-S1P, sphingosine and ceramide in the heart, serum and plasma were quantified as detailed in the Supplementary Materials.

2.3. Experimental I/R Injury

The heart was exposed and ischemia was achieved by ligating the left anterior descending coronary artery (LAD). After 30 minutes occlusion, reperfusion was achieved. After 24 hours of reperfusion the chest was re-opened and the LAD was occluded with the same suture. To estimate the ischemic area at risk (AAR), Evan's blue (3%) was injected into the left ventricle, circulated and uniformly distributed throughout the myocardium except AAR.¹⁶ The hearts were excised and transversely sliced. The heart sections were incubated with a 2% triphenyl tetrazolium chloride (TTC) for 10

minutes at 37 °C to stain viable myocardium. TTC-nonstained area (infarct area) was determined and expressed as infarct area/AAR (%).

2.4. Immunohistochemistry

Serial horizontal sections of formalin-fixed, paraffin-embedded hearts at the level of the maximal diameter were subjected to anti-SPHK1a immunohistochemistry using a rabbit polyclonal anti-SPHK1a antibody as described in Supplementary Materials.¹⁸ Aceton-fixed, fresh-frozen sections were also examined by indirect immunofluorescence by using a goat anti-desmin polyclonal antibody and a rabbit anti-SPHK1a antibody.

2.5. Determination of the activities of Rac1 and RhoA in cardiac tissue

Determinations of GTP-bound, active forms of Rac1 and RhoA in mouse hearts was performed by pull-down assay techniques as described previously⁹. Data are expressed as a percentage of the control value in the basal unstimulated state (= 100%).

2.6. Detection of Oxidative Stress Markers

The content of malondialdehyde, a major lipid peroxidation product, in heart tissues and

urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG), a stable marker of oxidative DNA damage, were determined by a spectrophotometrical method using BIOXYTECH MDA-586 assay kit (Oxis Research, Portland, OR, U.S.A.) and ELISA using an 8-OHdG ELISA assay kit (Nikken SEIL Corporation, Shizuoka, Japan)

2.7. Statistics

All data are shown as means $\pm$ SEM. ANOVA (analysis of variance) was followed by Dunnette's test to determine the statistical significance of differences between mean values. Unpaired *t*-test was performed for the comparison between two groups in Figs. 1B, 3E, 4B, 7, and 7B. For all statistical comparisons, $p < 0.05$ was considered significant.

3. Results

3.1. Expression and activity of SPHK1, and tissue and blood levels of S1P and related lipids in SPHK1 TG mice

We established two independent lines with different transgene expression levels as evaluated by Northern blot analysis (Supplementary Fig. 1). One of the TG lines,

designated as TG(H), showed widespread and high expression levels of the transgene transcript in a variety of organs including the heart, brain, kidney, stomach and other tissues. The other TG lineage, TG(L), showed a moderate or low level of transgene expression in the heart, testis and other tissues. TG(H) and TG(L) mice were both fertile and apparently normal, and grew comparably with WT mice. Measurements of sphingosine kinase enzymatic activity and Western blot analysis in the cardiac extracts confirmed functional overexpression of SPHK1 in the heart of both TG(H) and TG(L) mice (Fig. 1A). SPHK activity in the cytosolic fraction of the heart was 4-fold and 18-fold greater in TG(L) and TG(H) as compared to that in WT controls, respectively (Fig. 1A, *lower*). The heart tissues of TG(L) and TG(H) showed moderately and strongly elevated SPHK1 protein expression levels, respectively, which appeared comparable to respective SPHK activities, as compared with the WT heart (Fig. 1A, *upper*). Unexpectedly, however, the S1P content in the heart tissue was elevated by only 1.6-fold in TG(H), but not in TG(L), as compared to WT (Fig. 1B). We also measured the sphingosine and ceramide contents in the heart and found no difference between TG(H) and WT littermates (Fig. 1B). S1P concentrations in the plasma or serum were not significantly different between WT and TG(H) mice (Fig. 1C). Dihydro-S1P level in the serum were also similar between WT and TG(H) mice.

Immunofluorescent staining using anti-SPHK1 and anti-desmin antibodies demonstrated that SPHK1 was expressed in cardiomyocytes and other types of cells (Fig. 2A, *upper panels*). SPHK1 showed two expression patterns in WT cardiomyocytes, which included a faint homogenous pattern and a sparsely speckled pattern. These two patterns of staining by anti-SPHK1 antibody was not at all observed in the heart of SPHK1-knockout mice (Supplementary Fig. 3), indicating that the employed antibody specifically detected SPHK1 protein. In cardiomyocytes of TG(H) mice, SPHK1 expression was markedly elevated in both of these two patterns (Fig. 2A, *lower panels*). SPHK1 was also expressed in blood vessels of WT and TG(H) hearts, with similar expression levels (data not shown). RT-PCR analysis of a panel of key molecules of the S1P signaling system demonstrated that mRNA expression levels of S1P₁, S1P₂, and S1P₃ receptors and the enzymes, S1P phosphohydrolase 1 (SPP1), S1P lyase (SPL) and SPHK2, were not significantly different between WT and TG(H) hearts (Figs. 2B and 2C). Neither the heart rate, systemic blood pressure at resting, nor heart weight to body weight ratio were different between TG(H) mice and their WT littermates (Supplementary Table I). TG(L) mice also showed normal values of these parameters.

3.2. TG(H) Mice Develop Cardiac Fibrosis and Are Protected from I/R injury of

Myocardium

We found that both male and female TG(H) mice developed both interstitial and perivascular cardiac fibrosis, which were evident by 3 months and gradually progressed with aging (Figs. 3A and 3B). At 3 months of age none of the WT mice showed detectable pathological fibrosis in the heart. In the aged population (18 months or older), a small population of control WT mice (13%) also showed slight fibrosis, contrasting with the extensive fibrosis observed in 100% of TG(H) mice. TG(L) mice showed virtually no cardiac fibrosis until at least 9 months of age and were not different from WT mice. Fibrosis was observed in both the left and right ventricles of TG(H) hearts, and its distribution pattern was focal and scattered in nature. In most advanced cases fibrosis was transmural, in part, with marked thinning of the ventricular wall (Fig. 3A, *right*). Inspections with higher magnification revealed degenerative changes, which included intracellular small vacuoles and mild coagulative necrosis of cardiomyocytes (Fig. 3C). These changes were evident especially around the focal fibrotic lesions of the myocardium. Immunohistochemical analysis revealed scattered foci of extremely strong SPHK1 overexpression in the myocardium of TG(H) mice (Fig. 3D-c). The reason for this non-uniform expression pattern of the SPHK1 transgene product is unknown at present. High magnification inspection of serial sections processed for anti-SPHK1a

immunohistochemistry, Azan-, and HE-staining revealed that SPHK1a was expressed throughout the cytoplasm of affected cardiomyocytes with signs of mild degenerative changes, which were surrounded by collagen fibers, myocardial cells showing vacuolar degeneration, and a small numbers of necrotic cells (Fig. 3Dd-f). Thus, strong focal overexpression of SPHK1 protein was closely associated with degenerative changes and fibrosis. We also observed occasional calcification within the fibrotic areas. TUNEL-positive apoptotic cells were restricted to calcified regions (data not shown). We did not observe inflammatory cell infiltration in the heart nor did we find vessel abnormalities in the coronary vessels. We also found that TG(H) mice had normal blood biochemistry (Supplementary Table I) and blood cell counts including lymphocyte count (Supplementary Fig. 2).

Echocardiographic analysis of 3, 6, and 12 month old TG(H) mice revealed that most TG(H) mice showed normal left ventricular size with no thickness of the posterior wall or the interventricular septum, and they maintained the normal %FS (Supplementary Table II). However, we did observe one 9 month-old male mouse with dilated left ventricle and reduced %FS, which are echocardiographic signs reminiscent of human dilated cardiomyopathy.

Consistent with the pathological changes in TG(H) mouse hearts, they showed well

established molecular signs of cardiac remodeling, i.e., marked upregulation of the embryonic genes, including atrial natriuretic peptide (ANP) (3.12- (3 months (m)) and 3.12-fold (9 m) increases in TG(H) mice compared with WT mice), β -myosin heavy chain(β -MCH) (2.14- and 5.55-fold increases) and, to a lesser extent, brain natriuretic peptide (BNP) (1.13- and 1.72-fold increases) and skeletal muscle (SKM) α -actin (1.05- and 1.46-fold increases), as evaluated by Northern blot analysis (Supplementary Fig. 4). The mRNA expression levels of profibrotic factor TGF- β 1 and fibrosis-marker genes type I and III collagens, fibronectin and α -smooth muscle actin were not different between SPHK1 TG(H) and WT hearts (data not shown). The expression levels of collagen type I α 1 and α 2 proteins were elevated in the SPHK1-TG heart compared with WT heart (Supplementary Fig. 5).

We evaluated the effect of SPHK1 overexpression on I/R injury. Cardiomyocyte death in hearts (white area in Fig. 3E) exposed to 30 min of coronary occlusion and followed by 24 h of reperfusion was 30% reduced in TG(H) mice compared with WT mice. The ischemic area at risk (AAR) (white and red areas) was not different between the two mouse groups. Thus, TG(H) mice are protected from myocardial I/R injury.

3.3. Elevated Rac1 and RhoA Activities and Accumulation of Oxidative Stress Markers

in the Heart of TG(H) Mice

We and others have demonstrated in various types of cultured cells that S1P regulates Rac1 and Rho, which are implicated in ischemic and non-ischemic cardiac remodeling^{19,20}, in receptor subtype-specific manners.⁶ TG(H) heart tissues showed a 1.5-fold increase in the amount of GTP-bound, active form of Rac1 as compared to heart tissues obtained from WT littermates (Fig. 4A, *left*). By contrast, the total amount of Rac1 protein in the heart was similar between WT and TG(H) mice (Fig. 4A, *right*). The amount of GTP-bound RhoA in the TG(H) heart tissues was 1.3-fold greater compared to the WT (Fig. 6B, *left*). The total amount of RhoA protein was similar between TG(H) and WT heart (Fig. 4B, *right*).

Rac1 is involved in the generation of ROS in the heart,²⁰ which in concert with other mechanisms, contributes to cardiac remodeling. Indeed, TG(H) heart tissues showed a 1.5-fold increase in the content of malondialdehyde, which is a major lipid peroxide and a useful marker for tissue oxidative stress, as compared to WT littermates (Fig. 4C, *left*). Another oxidative stress marker, 8-OHdG, which is excised from oxidized DNA and secreted in the urine, showed a more than 2-fold increase over control (Fig. 4C, *right*). These results suggest that the hearts in TG(H) mice are exposed to increased oxidative stress as compared to those of WT littermates.

3.4. Successful Prevention of Cardiac Fibrosis in TG(H) Mice by the HMG CoA Reductase Inhibitor Pitavastatin and the Anti-oxidant N-2-Mercaptopropionylglycine (MPG)

In order to gain insight into the pathophysiology of SPHK1-mediated cardiac remodeling, we tested whether pitavastatin, which prevents membrane recruitment and activation of the Rho family GTPases,²¹ could exert a beneficial effect on TG(H) hearts. The administration of pitavastatin beginning at the age of 6 weeks after birth markedly reduced the extent of cardiac fibrosis evaluated at 12 week old, as compared to vehicle control (Figs. 5A and 5B). We also examined the effect of the anti-oxidant MPG,²² and found that MPG was effective in inhibiting development of cardiac fibrosis (Figs. 5A and 5B). By contrast, the angiotensin II type 1 receptor (AT₁) antagonist candesartan, which effectively inhibits pressure overload-induced cardiac hypertrophy and fibrosis, and another anti-hypertensive hydralazine, failed to inhibit fibrosis at doses that effectively and similarly lowered blood pressure (Figs. 5A and 5B).

3.5. S1P₃ Deletion Partially Inhibits Cardiac Fibrosis in TG(H) Mice

S1P₃ is the major S1P receptor subtype expressed in rodent cardiomyocytes and

fibroblasts.^{8, 10} Hence, we evaluated whether S1P₃ signaling was involved in cardiac fibrosis induced by SPHK1a overexpression, by generating S1P₃KO/TG(H) mice. Deletion of S1P₃¹³ resulted in a substantial reduction in the area of cardiac fibrosis as compared with S1P₃WT^(+/+)/TG(H) littermates (Figs. 6A and 6B), indicating that S1P₃ receptor signaling is at least partially involved in SPHK1-dependent cardiac remodeling and fibrosis. We examined activation of Smad3, which is a downstream effector of TGFβ and transactivated by S1P₃,^{23,24} in the heart. In the background of S1P₃^{+/+}, Smad3 phosphorylation in TG(H) heart was 50% increased compared with WT heart (Fig. 6C). Deletion of S1P₃ abolished an increase in Smad3 phosphorylation in TG(H) heart.

4. DISCUSSION

The present study demonstrates for the first time that chronic increased SPHK1 activity can induce cardiac fibrosis *in vivo*. Despite constitutive high enzymatic activity of SPHK1 in a variety of tissues including platelets, which have been believed to be one of major sources of plasma S1P,¹ SPHK1 TG(H) mice showed normal plasma and serum S1P levels, and only moderate increases in heart S1P level (Figs. 1B and 1C). These results strongly suggest the existence of a feedback regulation mechanism for

homeostasis of blood and tissue S1P levels *in vivo*. In the heart tissue, the levels of ceramide and sphingosine, the precursors of S1P, were not significantly different between WT and TG(H) mice, suggesting an increased turnover of sphingolipid metabolism in the transgenic heart, in which increased production of S1P by SPHK1 overexpression is balanced by increased degradation of S1P. Since mRNA expression levels of the S1P degrading enzymes, SPL and SPP1, were similar between WT and TG(H) mice (Figs. 2B and 2C), it is possible that these enzymes have a substantial intrinsic activity, or, alternatively, might be functionally upregulated by an excess amount of S1P, resulting in a homeostatically maintained S1P level that allows survival of mice despite increased SPHK activity. Particularly, it is an interesting possibility that SPL might play a major role in homeostasis of tissue and blood S1P levels in the face of increased SPHK activity, because a recent study²⁵ showed that deletion of SPL resulted in marked increases in tissue and blood S1P levels.

Chronic overexpression of SPHK1 resulted in the development of degenerative changes and interstitial fibrosis in the myocardium in the naïve heart, which advances with age (Fig. 3). Fibrosis was observed in 100% of the TG(H) hearts but not at all in TG(L) heart, indicating that cardiac fibrosis in SPHK1 TG mice was gene dose-dependent. Comparable myocardial changes were not observed in WT heart at the

same age throughout the observation period of 15 months, indicating that this cardiac fibrosis is not associated with normal aging. Despite degeneration and fibrosis, the cardiac function in most of TG(H) hearts was maintained at least until 12 months after birth (supplementary Table II), and the lifespan of the TG(H) mice were not shorter than the WT littermates (N. Takuwa, unpublished observation). However, we cannot exclude the possibility that TG(H) mice might develop diastolic dysfunction due to cardiac fibrosis because echocardiography is not optimal to detect diastolic dysfunction.²⁶ We observed that one 9 month old TG(H) mouse showed marked ventricular dilation reminiscent of human dilated cardiomyopathy. The ventricular dilation may have represented a decompensated stage of cardiac remodeling under sustained oxidative and possibly additional stresses in this SPHK1 TG(H) mice.

The development of SPHK1-mediated cardiac fibrosis was strongly inhibited in S1P₃ KO mice (Fig. 6). It is likely that S1P locally produced by overexpressed SPHK1 acts in a paracrine fashion on S1P₃, which is abundantly expressed in cardiomyocytes and fibroblasts among S1P receptors, and mediates cardiac fibrosis.^{10,14} The Smads, the major downstream signaling molecules of TGF- β , constitute the signaling pathways to induce tissue fibrosis through activating multiple downstream pathways.^{19,24} Deletion of S1P₃ abolished stimulation of Smad3 phosphorylation in the heart of SPHK1 TG mice

(Fig. 6C). It was recently demonstrated in several other cell types including fibroblasts, keratinocytes, and mesangial cells that the Smad signaling pathway is trans-activated by S1P receptors including S1P₃.^{23, 24} Particularly, S1P₃ stimulation in fibroblasts induced differentiation into myofibroblasts via trans-activation of Smad3.²³ The present observations together with these previous findings suggest the possibility that stimulation of Smad3 in SPHK1-TG heart may be brought about by trans-activation of TGF β signaling pathway by S1P-S1P₃ and involved in cardiac fibrosis. Further study is required to substantiate the role of the Smad pathway in S1P-S1P₃ mediated cardiac fibrosis. Our observation that TGF- β 1 mRNA level in the heart was not increased in SPHK1-TG mice compared with WT mice lends the support to the notion that Smad3 may be trans-activated in SPHK1-TG heart. It is possible that degradation process of collagens might be suppressed via S1P₃ receptor-mediated Smad trans-activation in the heart of SPHK1-TG mice as suggested in mesangial cells,²⁴ which is consistent with our observations that there was no increase in the mRNA levels of collagens and fibronectin in TG(H) heart.

As the molecular mechanisms for S1P-mediated cardiac fibrosis, we focused on Rho family GTPases, which contribute to cardiac fibrosis through more than a single mechanisms including ROS production, Rho kinase and NF- κ B activation¹⁹ and are

activated by S1P receptors in a receptor subtype-specific manner.⁶ We detected a modest increase in the activities of Rac and Rho in the TG(H) heart as compared to WT littermates (Fig. 4). Consistent with the notion that the Rho family GTPases are involved in the pathogenesis of cardiac fibrosis in the TG(H) heart, pitavastatin, which inhibits membrane targeting of both Rac1 and RhoA,²¹ strongly inhibited the development of fibrosis (Fig. 5). Rac is an upstream regulator essential for ROS production, which is implicated in cardiac fibrosis and remodeling.^{19, 20, 27} The levels of oxidative stress markers were increased in TG(H) mice (Fig. 4C). Moreover, the administration of the anti-oxidant MPG inhibited fibrosis (Fig. 5). It is reported that SPHK is also involved in ROS production in vascular smooth muscles.⁸ The morphological and pathophysiological analyses of SPHK1-TG mice (Supplementary Table I and other data) suggested that cardiac remodeling in the TG(H) heart was not secondary to hypertension, ischemic heart disease, or other metabolic disorders. These results collectively suggest that S1P₃-mediated Rho GTPase activation, Smad activation, and ROS production in the heart in concert result in cardiac fibrosis when S1P level in the heart is elevated. However, we cannot unequivocally preclude the possibility that the effects of SPHK1 overexpression in other tissues in part contribute to the observed cardiac phenotype indirectly through humoral or other mechanisms.

Spiegel and her colleagues²⁸ first proposed that SPHK1 and its product S1P act inside of cells to mediate mitogenic and anti-apoptotic effects. Recent studies^{29,30} showed in SPHK1^{-/-}SPHK2^{+/-} mice or SPHK1^{-/-} mice that marked accumulation of sphingosine and dihydrosphingosine, but not a decreased S1P level, resulted in increased cell death in the deciduum of pregnant mice and reduced tumor size in the spontaneous intestinal polyps, respectively. In the latter report,³⁰ deletion of the S1P₂ or S1P₃ receptor did not alter size of tumors. These observations suggested that intracellular accumulation of pro-apoptotic sphingosine, but not S1P receptor signaling, led to inhibition of cell proliferation or increased cell death. Our data rather suggest that cardiac fibrosis in SPHK1 TG mice is a S1P receptor-mediated event. Thus, the roles of sphingolipids in the regulation of cell survival and proliferation may be different, depending on cell types and the context of impairment of sphingolipid metabolism.

Recent investigations⁸ (for review) in the cardiovascular system have showed the cardio-protective role of the S1P signaling system in myocardial I/R injury. SPHK1 was suggested to be a key enzyme by the observation that deletion of SPHK1 aggravated I/R injury in an *in vitro* Langendorff apparatus.¹⁷ *In vivo* studies using S1P receptor-deleted mice^{15,16} showed that the cardio-protective effects of the S1P signaling system in ischemia/reperfusion injury models were mediated via S1P₃ or both S1P₃ and S1P₂.

Consistent with these previous investigations, the present study showed that chronic overexpression of SPHK1 in the mouse heart is cardio-protective against myocardial infarction due to I/R injury *in vivo* (Fig. 3E). Thus, SPHK1 overexpression has both the unfavorable fibrosis-promoting and favorable cardio-protective effects.

Immunofluorescent detection revealed two types of SPHK1 expression patterns, including a faintly homogenous pattern and a sparsely speckled pattern in both SPHK1-TG and WT mice (Fig. 2A). The speckled expression pattern may suggest that SPHK1 is present not only diffusely in the cytoplasm but also in the subcellular locations, most likely in the subcellular organella. In addition, the SPHK1 TG(H) mouse heart showed focal regions of strong overexpression of SPHK1 in the myocardium (Fig. 3D). At present, the reason for the latter expression pattern of the transgene product is not known. TG mice have been back-crossed to C57BL/6 mice more than 9 times, which excludes the possibility of genetic chimerism of the TG mice adopted in the present study. Whatever the reason for the uneven overexpression of the transgene product in the myocardium, our observation that severe pathological changes occur in the regions with SPHK1 overexpression strongly suggests that locally expressed SPHK1 is causally related to cardiomyocyte degeneration and fibrosis in the TG(H) heart.

In summary, the present study demonstrated for the first time that transgenic overexpression of SPHK1a in heart tissue led to chronic, progressive myocardial degeneration and fibrosis in mice while it conferred a protective effect on the myocardium against I/R injury. The SPHK1-dependent cardiac fibrosis in TG(H) mice likely involves the S1P₃-Rho family small G protein signaling pathway and downstream ROS production and subsequent TGF- β activation. These findings are consistent with the demonstration of other lysophospholipid receptors in fibrosis.³¹ The relevance of these observations in transgenic mice to human cardiac fibrosis of the ischemic, hypertensive, and other origins deserves further investigation.

Funding

This work was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Sciences, Sports and Culture of Japan (Y.T. and N.T.), and the NIH (DA019674 and NS048478) (J.C.).

Acknowledgments

We would like to thank Dr. R. Proia in National Institute of Diabetes and Digestive and

Kidney Diseases in U.S.A. for providing SPHK1-KO mice. We also thank Ms. Y. Ohta and C. Hirose for technical and secretarial assistance.

Conflict of interest

All authors have no potential conflict of interest.

References

1. Spiegel S, Milstien SJ. Functions of the multifaceted family of sphingosine kinases and some close relatives. *J Biol Chem* 2007; **282**:2125-2129.
2. Gardell SE, Dubin AE, Chun J. Emerging medicinal roles for lysophospholipid receptors. *Trends in Molecular Med* 2006; **12**:65-75.
3. Allende ML, Sasaki T, Kawai H, Olivera A, Mi Y, van Echten-Deckert G *et al*. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem* 2004;**279**:52487-52492.
4. Kharel Y, Lee S, Snyder AH, Sheasley-O'Neill SL, Morris MA, Setiady Y *et al*. Sphingosine kinase 2 is required for modulation of lymphocyte traffic by FTY720. *J Biol Chem* 2005; **280**:36865-36872.
5. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* 2005; **25**:11113-11121.
6. Takuwa Y. Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim Biophys Acta* 2002; **1582**:112-120.
7. Ishii I, Fukushima N, Ye X, Chun J. Lysophospholipid receptors: signaling and

biology. *Ann Rev Biochemistry* 2004;**73**:321-354.

8. Takuwa Y, Okamoto Y, Yoshioka K, Takuwa N. Sphingosine-1-phosphate signaling and biological activities in the cardiovascular system. *Biophys Biochim Acta* 2008;**1781**:483-488.

9. Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H *et al.* Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res* 2002; **90**:325-332.

10. Landeen LK, Aroonsakool N, Haga JH, Hu BS, Giles WR. Sphingosine-1-phosphate receptor expression in cardiac fibroblasts is modulated by in vitro culture conditions. *Am J Physiol* 2007;**292**:H2698-2711.

11. Robert P, Tsui P, Laville MP, Livi GP, Sarau HM, Bril A *et al.* EDG1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes, *J Mol Cell Cardiol* 2001;**33**:1589-1606.

12. Zhang J, Honbo N, Goetzl EJ, Chatterjee K, Karliner JS, Gray MO. Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia. *Am J Physiol* 2007;**293**: H3150-3158.

13. Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ *et al.* Selective loss

of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* 2001; **276**:33697-33704.

14. Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G *et al.* Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther* 2004, **309**:758-768.

15. Theilmeier G, Schmidt C, Herrmann J, Keul P, Schafers M, Herrgott I *et al.* High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* 2006;**114**: 1403-1409.

16. Means CK, Xiao CY, Li Z, Zhang T, Omens JH, Ishii I *et al.* Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *Am J Physiol* 2007;**292**:H2944-2951.

17. Jin ZQ, Zhang J, Huang Y, Hoover HE, Vessey DA, Karliner JS. A sphingosine kinase 1 mutation sensitizes the myocardium to ischemia/reperfusion injury. *Cardiovasc Res* 2007;**76**: 41-50.

18. Murate T, Banno Y, T-Koizumi K, Watanabe K, Mori N, Wada A *et al.* Cell

type-specific localization of sphingosine kinase 1a in human tissues. *J Histochem Cytochem* 2001; **49**:845-855.

19. Brown JH, Del Re DP, Sussman MA. The Rac and Rho hall of fame. A decade of hypertrophic signaling hits. *Circ Res* 2006;**98**:730-742.

20. Satoh M, Ogita H, Takeshita K, Mukai Y, Kwiatkowski DJ, Liao JK. Requirement of Rac1 in the development of cardiac hypertrophy. *Proc Natl Acad Sci U S A* 2006;**103**:7432-7437.

21. Laufs U, Kilter H, Konkol C, Wassmann S, Bohm M, Nickenig G. Impact of HMG CoA reductase inhibition on small GTPases in the heart. *Cardiovasc Res* 2002;**53**:911-920.

22. Sun JZ, Tang XL, Park SW, Qiu Y, Turrens JF, Bolli R. Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J Clin Invest* 1996;**97**:562-576.

23. Keller CD, Rivera Gil P, Tölle M, van der Giet M, Chun J et al. Immunomodulator FTY720 induces myofibroblast differentiation via the lysophospholipid receptor S1P3 and Smad3 signaling. *Am J Pathol* 2007;**170**:281-292.

24. Xin C, Ren S, Kleuser B, Shabahang S, Eberhardt W, Radeke H et al. Sphingosine 1-phosphate cross-activates the Smad signaling cascade and mimics transforming

growth factor- β -induced cell response. *J Biol Chem* 2004;**279**:35255-35262.

25. Vogel P, Donoviel MS, Read R, Hansen GM, Hazlewood J, Anderson SJ et al. Incomplete inhibition of sphingosine 1-phosphate lyase modulates immune system function yet prevents early lethality and non-lymphoid lesions. *PLoS ONE*. 2009;**4**:e4112.

26. Ouzounian M, Lee DS, Liu PP. Diastolic heart failure: mechanisms and controversies. *Nat Clin Pract Cardiovasc Med* 2008;**5**:375-586.

27. Sirker A, Zhang M, Murdoch C, Shah AM. Involvement of NADPH oxidase in cardiac remodeling and heart failure. *Am J Nephrol* 2007;**27**:649-660.

28. Olivera A, Kohama T, Edsall L, Nava V, Cuvillier O, Poulton S et al. Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J Cell Biol* 1999; **147**: 545-558.

29. Mizugishi K, Li C, Olivera A, Bielawski J, Bielawska A, Deng CX et al. Maternal disturbance in activated sphingolipid metabolism causes pregnancy loss in mice. *J Clin Invest* 2007; **11**:2993-3006.

30. Kohno M, Momoi M, Oo ML, Paik JH, Lee YM, Venkataraman K et al. Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation. *Mol Cell Biol* 2006;**9**:7211-7223.

31. Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z *et al.* The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med.* 2008;**14**:45-54.

Figure Legends

Fig. 1. Functional expression of SPHK1a in transgenic mice. (A) Functional expression of SPHK1a protein in TG(L) and TG(H) heart tissue. Cytosolic fractions of the heart homogenates (n=3) were subjected to *in vitro* SPHK assay. *Left*, The results are expressed as % of the control value obtained from WT mice. * $p < 0.05$, ** $p < 0.01$. *Right*, Western blot analysis of SPHK1a protein (indicated by an *arrowhead*) in the heart. (B) S1P, sphingosine, and ceramide contents in the heart of WT and TG(H) mice. Data represent the mean $\pm$ S.E. obtained from the numbers of animals shown in parenthesis at the bottom. ** $p < 0.01$ (C) S1P and dihydro-S1P (DH-S1P) levels in plasma and serum of WT and TG(H) mice.

Fig. 2. Expression of SPHK1 protein and related molecules in heart. (A) Immunofluorescent analysis of desmin (*red*) and SPHK1a (*green*) in cardiac tissues of WT (*upper panel*) and TG(H) (*lower panel*) mice. (B) Representative results of RT-PCR analysis performed in triplicate of the S1P₁, S1P₂, and S1P₃ receptors, SPHK1a, SPHK2, SPP, SPL. β -Actin was adopted as an internal control. (C) Quantification of the results in (B) expressed as relative mRNA expression levels, which were normalized to β -actin.

Fig. 3. Development of cardiac fibrosis in TG(H) mice. (A) Representative photographs of Azan-stained horizontal sections of the heart from 3, 9, and 15 month old TG(H) mice. *Inset*, magnification of a region of transmural fibrosis. (B) Quantified data of cardiac fibrosis. Four or five mice in each group were analyzed. (C) HE-stained section of a TG(H) mouse heart, showing vacuolar changes (*arrows*) and mild coagulative necrosis (*arrowheads*) of cardiomyocytes around the fibrotic area (*asterisks*). Cardiac fibrosis was not observed in TG(L) mice until the age of 9 months. (D) Comparison of immunohistochemical staining of the SPHK1 protein (brown color, a and c) and fibrosis in Azan staining (blue color, d and f) and HE staining (e) in a TG(H) mouse heart. (E) Myocardial infarct in WT and TG(H) mice after ischemia/reperfusion (I/R). *Upper*: representative photomicrographs of ventricles. Blue areas, non-ischemic tissue; white areas circumscribed with the red line, infarcted area; red areas circumscribed with the yellow line except white area, salvaged tissues. The red and white areas are the area at risk (AAR). *Lower*: quantified infarct areas corrected for AAR. $**p < 0.01$

Fig. 4. TG(H) heart tissue showed increased activities in Rac and RhoA small GTPases and increased levels of oxidative markers. The amounts of GTP-bound,

active forms of Rac (A) and RhoA (B) in the heart of WT and TG(H) mice were determined by pull-down assay technique and normalized by total GTPase amounts. $*p<0.05$, $**p<0.01$. Total Rac and RhoA protein levels were not different from WT control. (C) TG(H) mice showed increased levels of oxidative stress markers. Malondialdehyde content in the heart tissue (*left*) and urinary secretion of 8-OHdG (normalized by creatinine concentration, *right*) were quantified and compared between TG(H) and WT mice. Data represent the mean $\pm$ S.E. obtained from 5 to 9 mice. $*p<0.05$, $**p<0.01$.

Fig. 5. Preventive effects of an HMG CoA reductase inhibitor (pitavastatin) and an anti-oxidant (MPG), but not an angiotensin II type 1 receptor antagonist (TCV116), against development of cardiac fibrosis. (A) Representative photographs of Azan stained horizontal sections of the heart obtained from TG(H) mice that received pitavastatin, MPG, TCV-116, hydralazine, or vehicle. (B) Quantitative analysis of fibrotic area expressed as the percentage of the total cross sectional area. TG(H) mice at the age of 6 weeks were randomly assigned to either of the treatment groups, each consisting of 5 to 7 mice. Medical intervention was continued until 12 weeks old. Hydralazine, which was employed as a control for TCV116 that causes a decrease in

blood pressure, did not have any effect on fibrosis by itself. $**p < 0.01$.

Fig. 6. Deletion of the S1P₃ receptor reduced the development of cardiac fibrosis and stimulated Smad3 phosphorylation in TG(H) mice. (A) Representative photographs of Azan stained horizontal sections of the heart obtained from WT/TG(H) or S1P₃KO/TG(H) mice. (B) Quantitative analysis of the fibrotic area expressed as a percentage of the total cross sectional area. Littermate S1P₃WT/TG(H) and S1P₃KO/TG(H) mice (n=5) at the age of 16 weeks were subjected to analysis. (C) Western blot analysis of Smad3 phosphorylation. The upper and lower panels show phosphorylated Smad3 (p-Smad3) and total Smad2/3, respectively. The bands indicated by the asterisks on the blots most likely represent non-specific or degradation products of total Smad2/3 and p-Smad3. $*p < 0.05$.

Supplementary Materials

Materials and Methods

Generation of SPHK1 Transgenic (SPHK1 TG) Mice and S1P₃ knock-out (KO)/SPHK1 TG Bigenic Mice

SPHK1 TG mice that overexpress SPHK1a isoform under the universal CAG promoter was generated as described previously¹. TG mice were back-crossed to C57BL/6J more than 9 times. SPHK1 TG mice was identified by genotyping with the PCR using the forward PCR primer 5'-AGGGGCGTGTTTCTGTGGAT-3' and the reverse primer 5'-TGGCAGAGGGAAAAAGATCTC-3'. PCR comprised 30 cycles of 0.5 min at 94°C, 0.5 min at 60°C, and 0.5 min at 72°C. The PCR product consisted of 261 bp. S1P₃ knock-out (KO) mice in C57BL/6 genetic background were described previously². Matings between S1P₃ KO and SPHK1 TG mice resulted in generation of S1P₃^{+/-}/SPHK1TG and S1P₃^{+/-}/WT siblings with the expected 1:1 ratio. S1P₃^{+/-}/SPHK1TG and S1P₃^{+/-}/WT siblings were then crossed to obtain S1P₃^{+/+}/SPHK1TG and S1P₃^{-/-}/SPHK1TG littermates. The mice were housed in conventional conditions with free access to water and regular food in an air-conditioned room.

Northern Blot analysis and RT-PCR analysis

Total RNA was obtained from the cardiac apex as previously described³. The expression level of SPHK1 mRNA was analyzed by Northern blot analysis using a 459 bp *EcoRI-HinCII* fragment of pCAGGS-SPHK1a¹ as a probe as described previously³. Probes for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β -myosin heavy chain (β -MHC), skeletal muscle (SKM) α -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control were obtained as described³. The radioactivities of corresponding bands were quantified by using Fuji BAS bioimage analyzer 2000 (Fuji Film, Tokyo, Japan) and expressed as a fold-increase above the control which is expressed as 1.0. RT-PCR analysis of S1P₁, S1P₂, S1P₃, and SPHK1 were performed as described by Ryu et al.⁴, that of SPHK2 was performed as described by Mizugishi et al.⁵, and SPP and SPL according to the method described by Pettus et al.⁶. The forward and reverse primers for TGF- β 1 were 5'-CCAAAGACATCTCACACAGTA-3' and 5'-TGCCGTACAACCTCCAGTGAC-3'.

Western Blot Analysis

For detection of SPHK1a protein in the heart, the homogenates were prepared in ice-cold buffer A containing 250 mM sucrose, 10 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na₃VO₄, 20 mM NaF, 1 mM phenylmethylsulphonylfluoride, and 20 mg/ml each of leupeptin and aprotinin. After

centrifugation at 3,000 x g the supernatant devoid of nuclei and debris were centrifuged at 10,000 x g for 10 min to obtain a supernatant post-mitochondrial fraction, which was further centrifuged at 100,000 x g for 60 min at 4 °C to separate cytosolic and microsomal fractions. Cytosolic protein was dissolved in Laemmli's sample buffer followed by 10% SDS-polyacrylamide gel electrophoresis and Western blot analysis using a rabbit polyclonal anti-SPHK1a as described⁷. Rabbit polyclonal phospho(Ser423/425)-Smad3 antibody (#9514) and mouse monoclonal Smad2/3 antibody (#610843) were purchased from Cell Signaling and BD BioSciences, respectively. In Western analysis of phospho-Smad3, the primary and secondary antibodies was diluted in Can Get Signal-Solution 1 and -Solution 2 (TOYOBO, Osaka, Japan), respectively, followed by visualization using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockland, IL, U.S.A.). For detection of procollagen type I α 1 and α 2 polypeptides, the cardiac homogenates (90 μ g protein) prepared by using the buffer A was separated on 8% SDS-PAGE, followed by detection using goat polyclonal anti-type I α 1 (#sc-8783) and type I α 2 (#sc-8786) antibodies (Santa Cruz).

Measurements of SPHK Activity and Levels of S1P, Dihydro-S1P, Sphingosine, and

Ceramide

SPHK activity in the heart and other tissues was measured as described previously⁸. In brief, the cardiac apex was dissected, rinsed in ice-cold phosphate-buffered saline, snap frozen in liquid nitrogen, and then homogenized in ice-cold sphingosine kinase buffer which contained 50 mM Tris (pH 7.5), 10 % glycerol, 1 mM β -mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β -glycerophosphate, 15 mM NaF, 10 μ g/ml each of leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine. After centrifugation at 10, 000 x g, the supernatant was ultracentrifuged at 100, 000 x g for 30 min to obtain the cytosolic fraction. Protein (15 μ g) was incubated with 50 μ M sphingosine (prepared in mixed micelles in the presence of Triton X-100), 10 μ Ci of [γ -³²P]ATP (1 mM) and 10 mM MgCl₂. Labeled lipids were extracted and resolved by thin-layer chromatography as described previously⁸.

S1P content in the heart, serum and plasma, and sphingosine content in the heart were quantified as described previously⁹. The heart tissue was homogenized in 1 M NaCl-25 mM HCl solution and extracted by sequentially adding equal volumes of methanol and chloroform, and 1/10 volume of 3 N NaOH. After phase separation, the organic phase was re-extracted with methanol-1 M NaCl (1:1, vol/vol). Mass levels of S1P and sphingosine, which were recovered in combined aqueous and organic fractions,

respectively, were measured⁵.

Levels of S1P and dihydro-S1P (DH-S1P) in the plasma and serum were also quantified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using Voyager System 4058 (Applied Biosystems) as described¹⁰, with similar results. DH-S1P was detected at m/z 968. However, second stable isotopic ion of S1P, (M+2) ion of S1P, appears at the same mass (m/z 968). Therefore, actual intensity of DH-S1P was calculated by subtracting the intensities of (M+2) ion of S1P. The amounts of DH-S1P were determined based on the ratio between its actual intensity and the intensity of C17 S1P as an internal standard. Quantitative measurement of ceramide species was made on lipid extracts obtained by the methods of Bligh and Dyer, using a triple-quadrupole mass spectrometer (Finnigan MAT TSQ 7000) as previously described¹¹. Each sample was analyzed in duplicate.

Measurements of Heart Rates and Blood Pressure in Conscious Mice

Heart rate and systolic and diastolic blood pressure of conscious mice were measured by a tail cuff method using Softron BP98A (Softron Co. Tokyo)¹. Prior to the measurements, the mice were trained to become accustomed to the tail cuff method by undergoing trial sessions in one week. At least seven measurements were performed on individual mice to obtain the mean values. Before each experiment mice were kept at 37

°C for 10 min to avoid any vasoconstriction caused by environmental stress.

Echocardiography

Mice were anesthetized with pentobarbital (60 mg/kg) and echocardiography was performed using an ultrasonography (Agilent Technology SONOS5500). A 12-MHz linear ultrasound transducer (S12) was applied to the depilated left anterior chest wall. M-mode measurements of left ventricular (LV) internal diameters, interventricular septal thickness, and posterior wall thickness were taken for more than five beats and averaged. LV end-diastolic diameter (LVEDD) was measured at the time of apparent maximal LV diastolic dimension. LV end-systolic diameter (LVESD) was measured at the time of the most anterior systolic excursion of the posterior wall. Percent fractional shortening (%FS) was calculated as $(LVEDD - LVESD) / LVEDD \times 100$.

Experimental Ischemia/Reperfusion (IR) Injury and Myocardial Infarction

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (65mg/kg). A positive-pressure respirator was used with 65% oxygen during the surgical procedure. Mice were kept warm using heating pads. The heart was exposed by left thoracotomy between the ribs (third intercostals space). Ischemia was achieved by ligating the left anterior descending coronary artery (LAD) using a 7-0 nylon suture with a 1mm silicon

tubing placed on top of LAD. Myocardial ischemia was confirmed by ECG change (ST elevation). After 30 minutes occlusion, the silicon tubing was removed to achieve reperfusion and hearts were harvested after 24 hours of reperfusion. After I/R, mice were reanesthetized and intubated, and the chest was opened. The LAD was occluded with the same suture, which had been left at the site of the ligation. To estimate the ischemic area at risk (AAR), Evan's blue (3%) was injected into the left ventricle, circulated and uniformly distributed without risk area.¹² After arresting the heart at the diastolic phase by KCl injection, hearts were quickly excised and sliced into 1-mm thick cross sections. The heart sections were incubated with a 2% triphenyl tetrazolium chloride for 10 minutes at 37 degree. TTC stained area (ischemic and viable area), TTC negative stained area (infarct area), and total LV area from both sides of each section were measured using Image J (NIH, USA), and each section was multiplied by the weight of the section and then totaled from all sections. AAR/LV, infarct area/LV, and infarct area/AAR were expressed as a percentage.

Histological Analysis, Immunohistochemistry, and Immunofluorescence

Wild-type mice, SPHK1-TG mice, and SPHK1-KO mice (kindly supplied by Dr. Richard Proia in National Institute of Diabetes and Digestive and Kidney Diseases,

Bethesda, MD, U.S.A.) were euthanized by intraperitoneally injecting an excess dose of pentobarbital. Serial horizontal sections of formalin-fixed, paraffin-embedded hearts at the level of the maximal diameter were subjected to anti-SPHK1a immunohistochemistry, Azan staining (for detection of fibrosis), and HE staining. Immunohistochemical detection of SPHK1a was performed using a rabbit polyclonal anti-SPHK1a antibody as described previously⁷. Aceton-fixed, fresh-frozen sections were also examined by indirect immunofluorescence by using a goat anti-desmin polyclonal antibody and a rabbit anti-SPHK1a antibody.

Determination of the Activities of Rac1 and RhoA in Cardiac Tissue

Determinations of GTP-bound, active forms of Rac1 and RhoA in the mouse heart was performed by pull-down assay techniques as described previously¹⁴. In brief, the heart homogenates were prepared in an ice-cold lysis buffer and 10,000 x g supernatant was incubated at 4 °C with either GST-PAK (for active Rac1) or GST-rhotekin (for active RhoA) immobilized on glutathione-Sepharose beads for 40 min. The proteins bound to Sepharose beads, as well as total Rac1 or RhoA derived from a portion of the same cell lysate, were solubilized with Laemmli's sample buffer and subjected to electrophoresis and Western blot analysis, using mouse monoclonal anti-Rac1 and anti-RhoA antibodies

as described¹³. The corresponding bands were densitometrically quantitated by using the Quantity One Image Analyzing System (Bio-Rad). Data are expressed as a percentage of the control value in the basal unstimulated state (= 100%).

Detection of Oxidative Stress Markers

The content of malondialdehyde, a major lipid peroxidation product, in heart tissue was spectrophotometrically quantitated by using BIOXYTECH MDA-586 assay kit (Oxis Research, Portland, OR) according to manufacturer's instructions. Urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG), a stable marker of oxidative DNA damage, was measured in the urine by using an 8-OHdG ELISA assay kit (Nikken SEIL Corporation, Shizuoka, Japan), according to the manufacturer's instructions. The results were normalized on the basis of creatinine concentration that was measured by Jaffe reaction.

Chronic Treatment of Mice with Pharmacological Agents

Six week-old TG(H) and WT littermates were randomized to 6 weeks of treatment with either of the followings: pitavastatin (1 mg/kg/day in drinking water) kindly donated from the Kowa Pharmaceutical Company (Nagoya, Japan), MPG (100 mg/kg/d by daily i.p. injections) (Sigma)¹⁴, their vehicle control (Dulbecco's phosphate buffered saline),

TCV-116 (10 mg/kg/day in drinking water) generously supplied by Takeda Pharmaceutical Company (Osaka, Japan), its hypotensive control, hydralazine (10 mg/kg/day in drinking water) (Sigma). After treatment the mice were euthanized by an excess dose of pentobarbital and the extent of cardiac fibrosis was analyzed using the NIH Image J software.

Statistics

All data are shown as mean $\pm$ SEM. ANOVA (analysis of variance) was followed by Dunnett test to determine the statistical significance of differences between mean values. Unpaired *t*-test was performed for the comparison between two groups in Figs. 1B, 4B, 7, and 7B. For all statistical comparisons, $p < 0.05$ was considered significant.

References

1. Oyama O, Sugimoto N, Qi X, Takuwa N, Mizugishi K, Koizumi J, Takuwa Y. The lysophospholipid mediator sphingosine-1-phosphate promotes angiogenesis in vivo in ischaemic hindlimbs of mice. *Cardiovasc Res.* 2008;78:301-307.
2. Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J. Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled

- receptor, LP(B3)/EDG-3. *J Biol Chem.* 2001; 276:33697-33704.
3. Usui S, Sugimoto N, Takuwa N, Sakagami S, Takata S, Kaneko S, Takuwa Y: Blood lipid mediator sphingosine 1-phosphate potently stimulates platelet-derived growth factor-A and -B chain expression through S1P1-Gi-Ras-MAPK-dependent induction of Kruppel-like factor 5. *J Biol Chem* 2004, 279:12300-12311.
 4. Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Y Takuwa: Sphingosine-1-Phosphate, a Platelet-Derived Lysophospholipid Mediator, Negatively Regulates Cellular Rac Activity and Cell Migration in Vascular Smooth Muscle Cells. *Circ Res* 2002, 90:325-332.
 5. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL: Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* 2005, 25:11113-11121.
 6. Pettus BJ, Bielawski J: The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J* 2003, 17:1411-1421.
 7. Murate T, Banno Y, T-Koizumi K, Watanabe K, Mori N, Wada A, Igarashi Y, Takagi A, Kojima T, Asano H, Akao Y, Yoshida S, Saito H, Nozawa Y: Cell type-specific localization of sphingosine kinase 1a in human tissues. *J Histochem Cytochem* 2001,

49:845-855.

8. Olivera A, Barlow K, Spiegel S: Assaying sphingosine kinase activity. *Methods Enzymol* 2000, 311:215-223.
9. Edsall L, Vann L, Milstien S, Spiegel S: Enzymatic method for measurement of sphingosine 1-phosphate. *Methods Enzymol* 2000, 312:9-16.
10. Tanaka T, Tsutsui H, Hirano K, Koike T, Tokumura A, Satouchi K: Quantitative analysis of lysophosphatidic acid by time-of-flight mass spectrometry using a phosphate-capture molecule. *J Lipid Res* 2004, 45:2145-2150.
11. Ichi I, Nakahara K, Miyashita Y, Hidaka A, Kutsukake S, Inoue K, Maruyama T, Miwa Y, Harada-Shiba M, Tsushima M, Kojo S: Association of ceramides in human plasma with risk factors of atherosclerosis. *Lipids* 2006, 41:859-863.
12. Means CK, Xiao CY, Li Z, Zhang T, Omens JH, Ishii I *et al.* Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *Am J Physiol* 2007;**292**:H2944-2951.
13. Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y: Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol Cell Biol* 2000, 20:9247-9261.

14. Yamamoto M, Yang G, Hong C, Liu J, Holle E, Yu X, Wagner T, Vatner SF, Sadoshima J: Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy. J Clin Invest 2003, 112:1395-1406

Supplemental Tables

Table I. Heart/ body weight, heart rate, blood pressure, and blood biochemistry

	WT		SPHK1 Tg(H)	
Heart/ BW (mg/g)	4.88±0.13	(8)	4.83±0.19	(7)
Heart rate (beats/min)	644±13	(8)	637±15	(7)
Systolic BP (mmHg)	97±2	(8)	101±3	(7)
Diastolic BP (mmHg)	56±2	(8)	59±3	(7)
Serum total chol (mg/dl)	86.0±13.4	(4)	75.3±3.8	(3)
Serum triglyceride (mg/dl)	82.7±24.1	(4)	65.6±19.3	(3)
Serum glucose (mg/dl)	187±16	(4)	209±28	(3)

20 weeks old male SPHK1 Tg (H) and wild-type (WT) littermates were analyzed.

The values are means±S.E. BW, body weight; BP, blood pressure; chol, cholesterol.

The numbers in the parentheses indicate the numbers of analyzed mice.

Table II. Echocardiographic analysis of WT and SPHK1-Tg (H) hearts

	3 months		6 months		12 months	
	WT	TG	WT	TG	WT	TG
	n=8	n=5	n=6	n=6	n=6	n=3
EDD	3.27±0.04	3.29±0.04	3.44±0.07	3.39±0.10	3.62±0.02	3.58±0.07
ESD	2.10±0.02	2.09±0.04	2.16±0.06	2.19±0.07	2.35±0.02	2.31±0.07
%FS	35.7±0.85	36.5±1.11	37.1±2.16	35.2±2.33	35.1±0.78	35.0±2.71
IVSD	0.63±0.02	0.63±0.01	0.73±0.02	0.72±0.02	0.76±0.01	0.77±0.01
IVSS	0.77±0.03	0.79±0.02	0.86±0.02	0.85±0.02	1.10±0.06	1.06±0.02
PWD	0.63±0.02	0.63±0.01	0.68±0.03	0.65±0.06	0.71±0.03	0.75±0.03
PWS	0.79±0.02	0.80±0.02	0.89±0.02	0.88±0.02	0.98±0.05	1.03±0.06

Mice of indicated ages were subjected to echocardiographic analysis. LVEDD, left ventricular end diastolic diameter, LVESD, left ventricular end systolic diameter; %FS, percent fractional shortening; IVSS, interventricular septal thickness in systole; IVSD, interventricular septal thickness in diastole; PWD, posterior wall thickness in diastole; PWS, posterior wall thickness in systole. Data are expressed as means ± S.D. Numbers of mice in each group are given in parenthesis. ns, not significant. EDD, ESD, IVSD, IVSS, PWD and PWS are expressed in millimeters. The values are means±S.E.

Legends of supplemental figures

Supplemental Fig. 1. Functional expression of SPHK1a in transgenic mice. (A)

Different mRNA expression patterns of the SPHK1a transgene (indicated by *arrowheads*) in two lines of SPHK1aTG mice, TG(L) and TG(H). Total RNA (15 µg) prepared from various organs of TG(L) and TG(H) mice were subjected to Northern blot analysis. TG mice of both lineages showed an identical expression pattern for endogenous SPHK1a mRNA (*arrows*) as in WT mice, with high expression levels in the lung, spleen, kidney, intestine, uterus, and skin. The results for transient SPHK1a overexpression in mouse B16 melanoma cells and parental cells are also shown (*right*).

Supplemental Fig. 2. TG(H) mice showed normal blood cell count.

Both wild type (WT) and TG(H) littermate mice (three mice each) were analyzed for numbers of peripheral blood red blood cells (RBC), white blood cells (WBC), lymphocytes (Lymph), and platelets (Plt). TG(L) mice showed normal values.

Supplemental Fig. 3. Immunofluorescent staining of SPHK1a (*green*) in cardiac

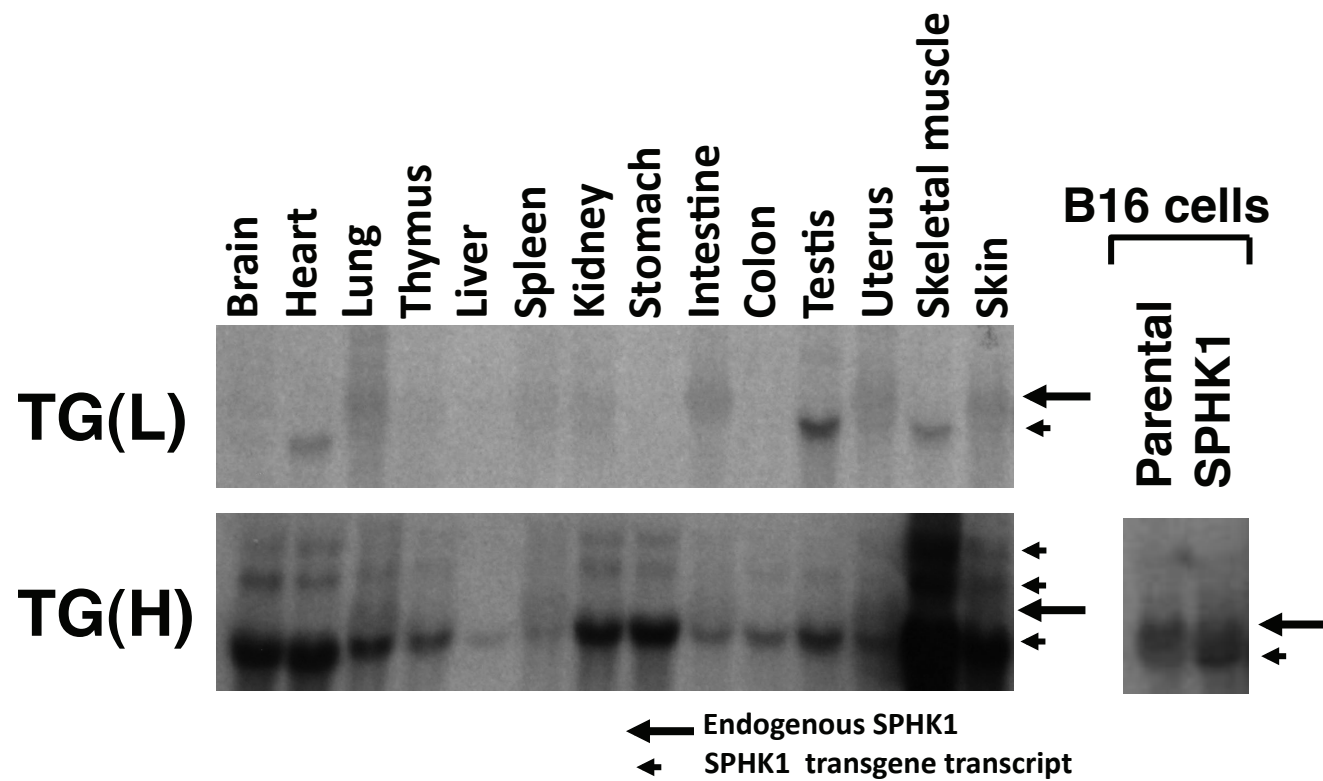
tissues of WT (*left panel*) and SPHK1-KO (*right panel*) mice. Cryosections of the

hearts from wild-type and SPHK1-homozygouslydisrupted (SPHK1-KO) mice were

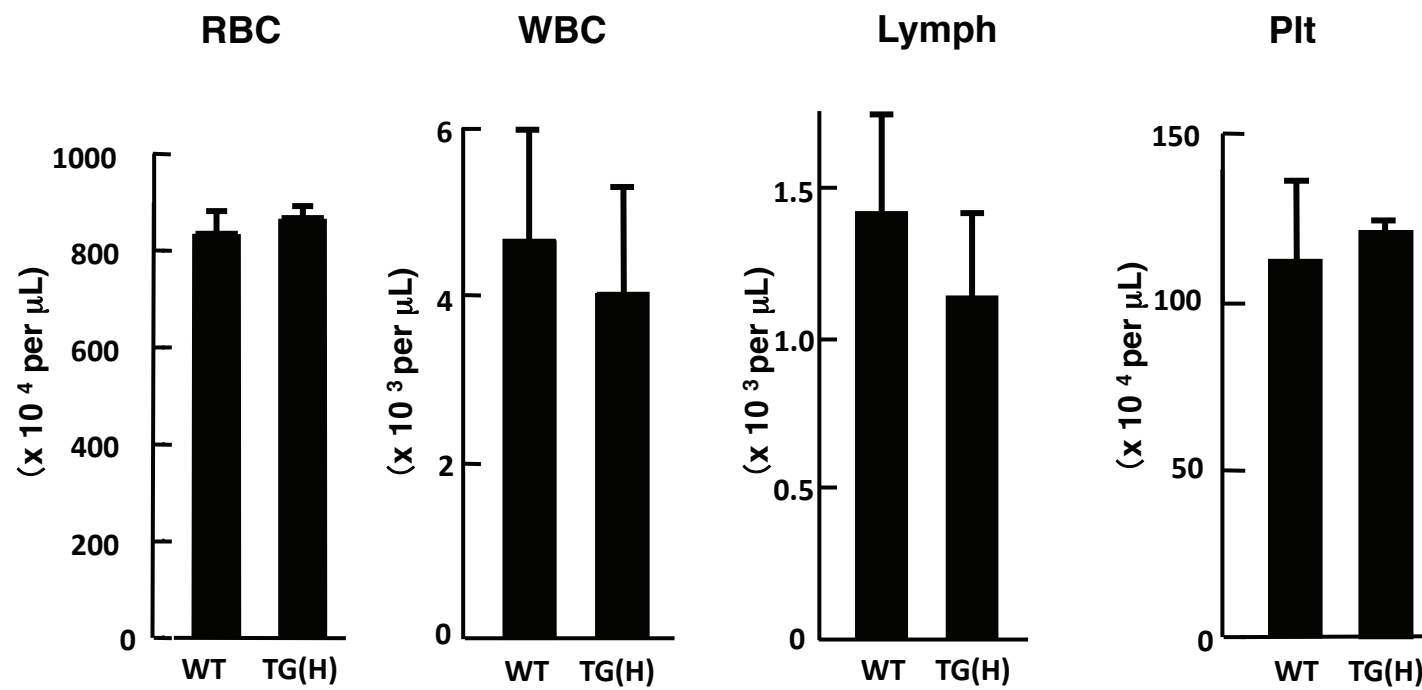
stained with anti-SPHK1-specific antibody as described in the Supplementary Materials and Methods. Cell nuclei were stained with DAPI.

Supplemental Fig. 4. Upregulation of embryonic genes and profibrotic genes in TG(H) hearts at the ages of 3 and 9 months. (A) Expression of “fetal” genes. Total RNA prepared from ventricles of WT and TG(H) mice (n=3 or 4) were analyzed for the expression of mRNAs of SPHK1, ANP, BNP, β -MHC, and SKM α -actin by Northern blot. GAPDH was used as an internal standard.

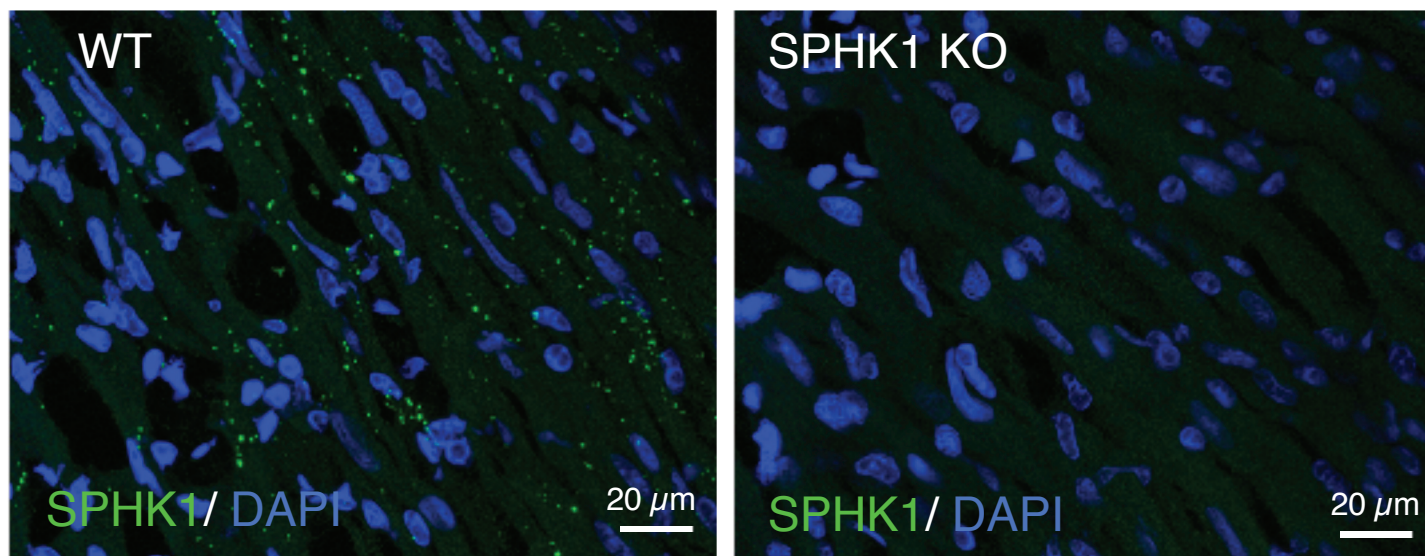
Supplemental Fig. 5. Upregulation of the expression of collagen type I α 1 and α 2 polypeptides. The homogenates of the hearts from wild-type and SPHK1-TG(H) mice were prepared as described in the Supplementary Materials and Methods were analyzed, and analyzed Western blotting using specific anti-procollagen type I α 1- and α 2-antibodies.



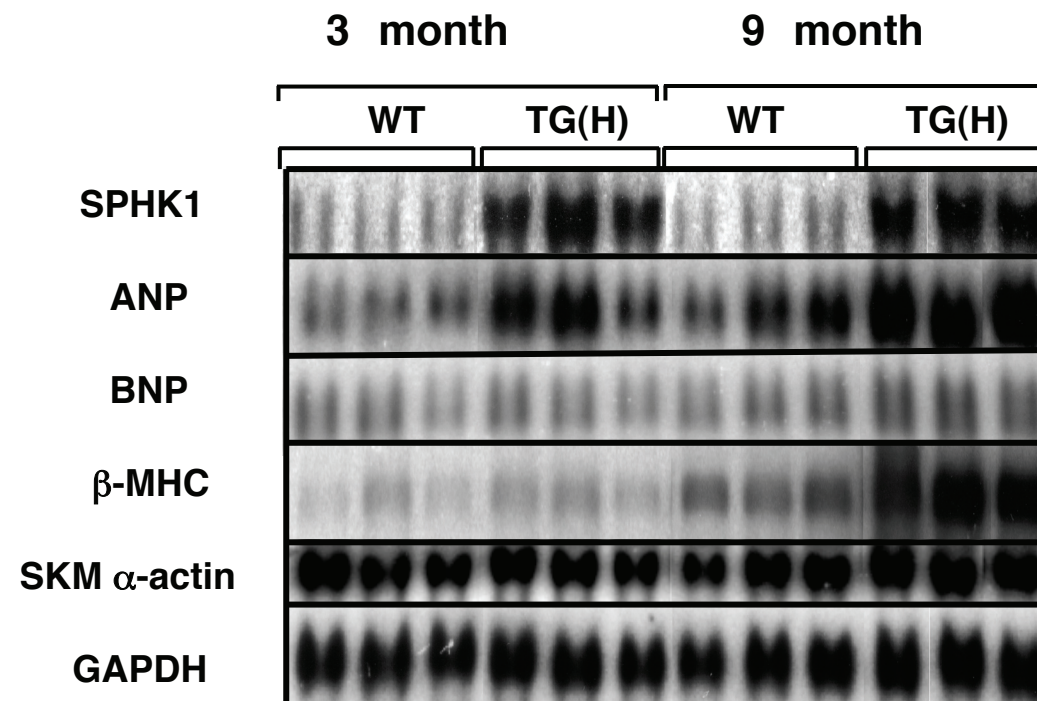
Supple. Fig. 1



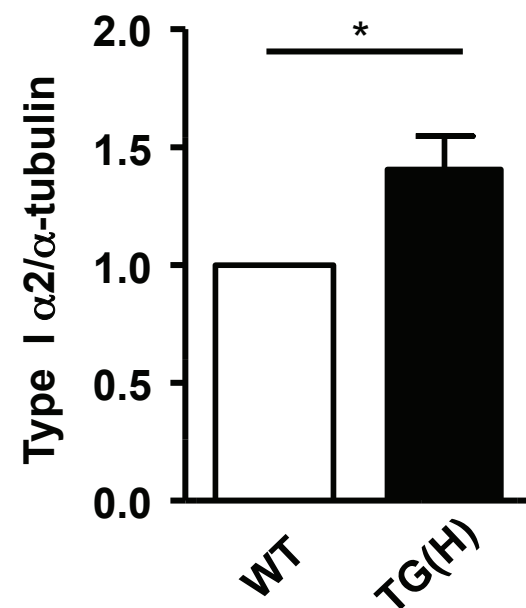
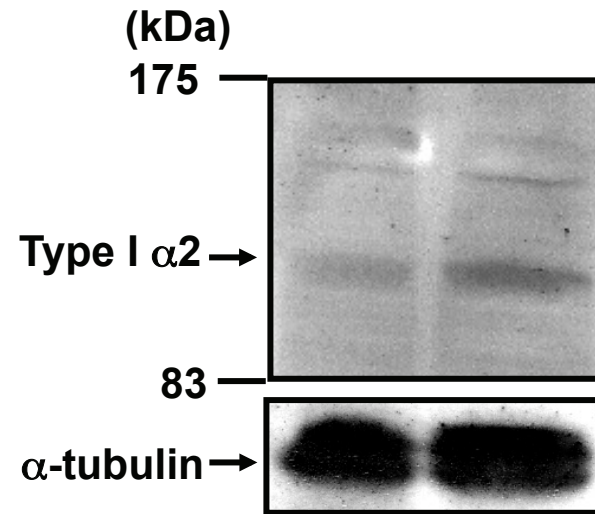
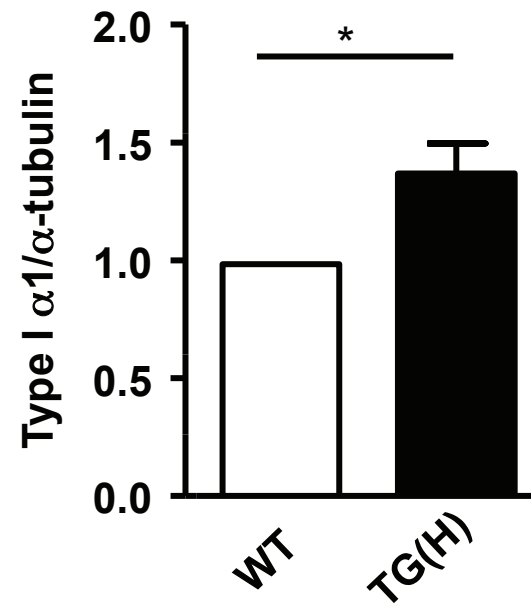
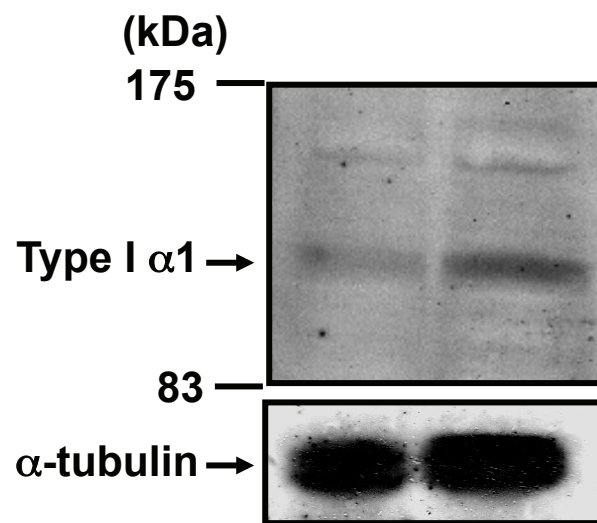
Suppl. Fig. 2



Suppl. Fig. 3



Suppl. Fig. 4



Suppl. Fig. 5

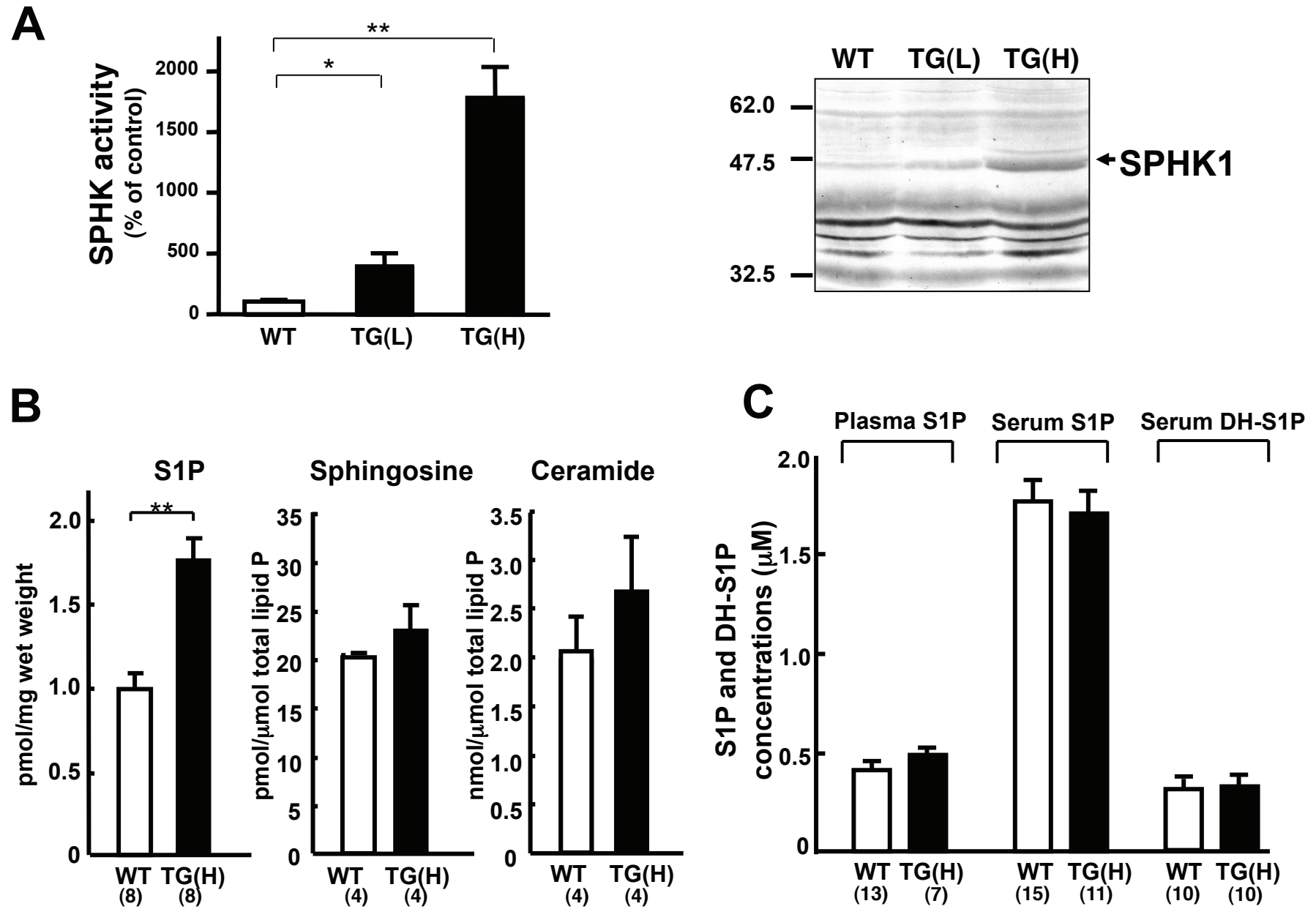


Fig. 1

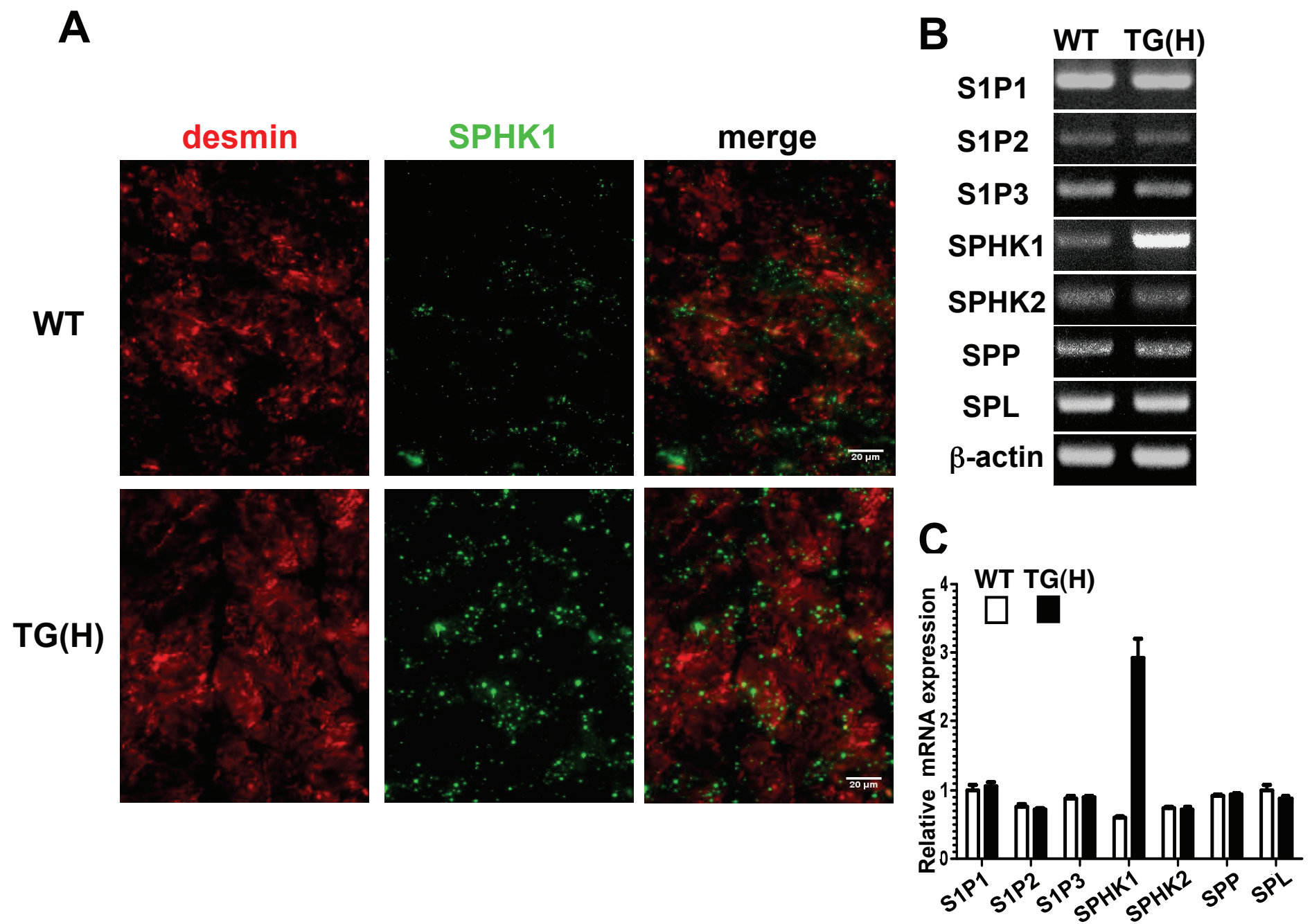


Fig. 2

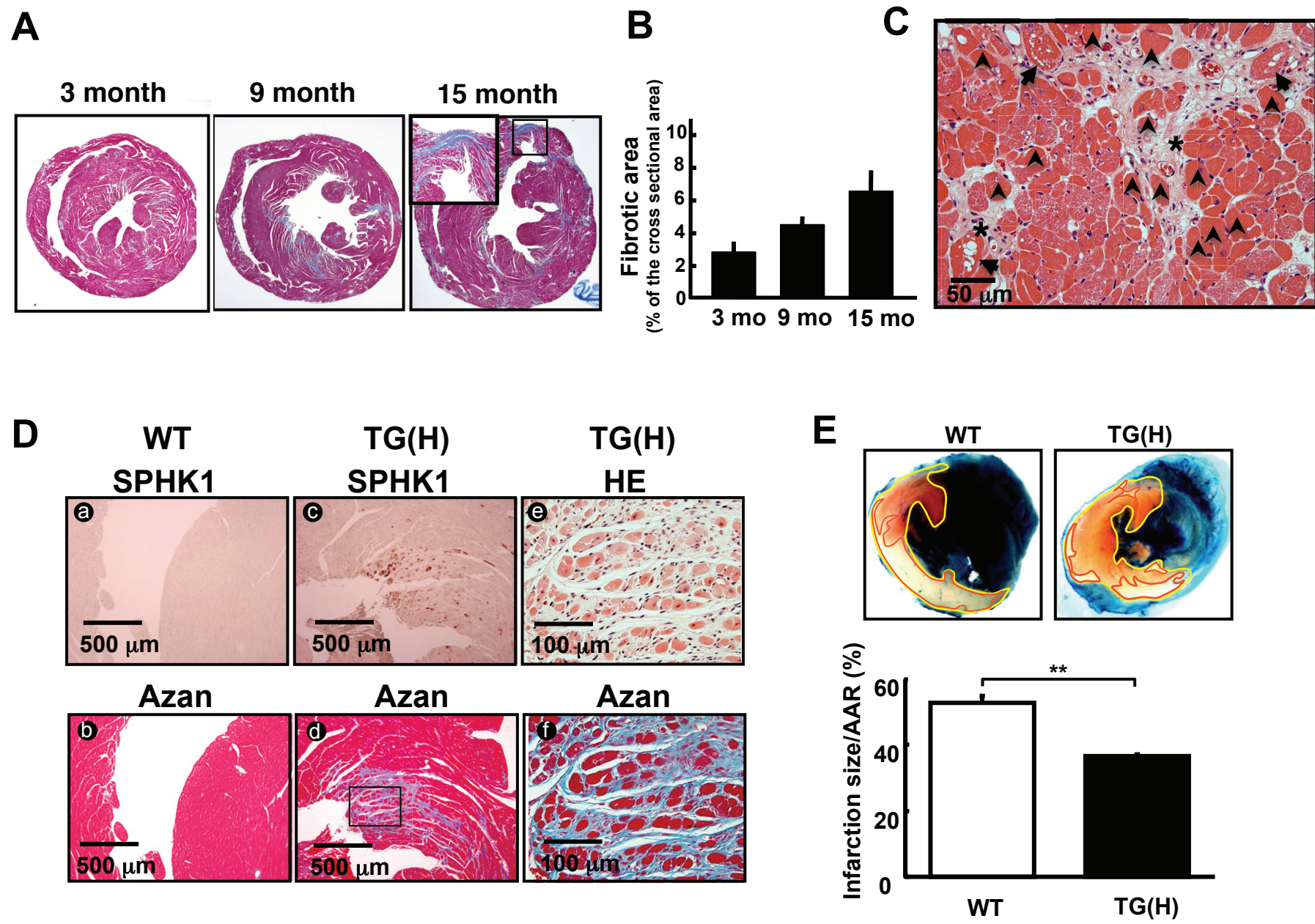


Fig. 3

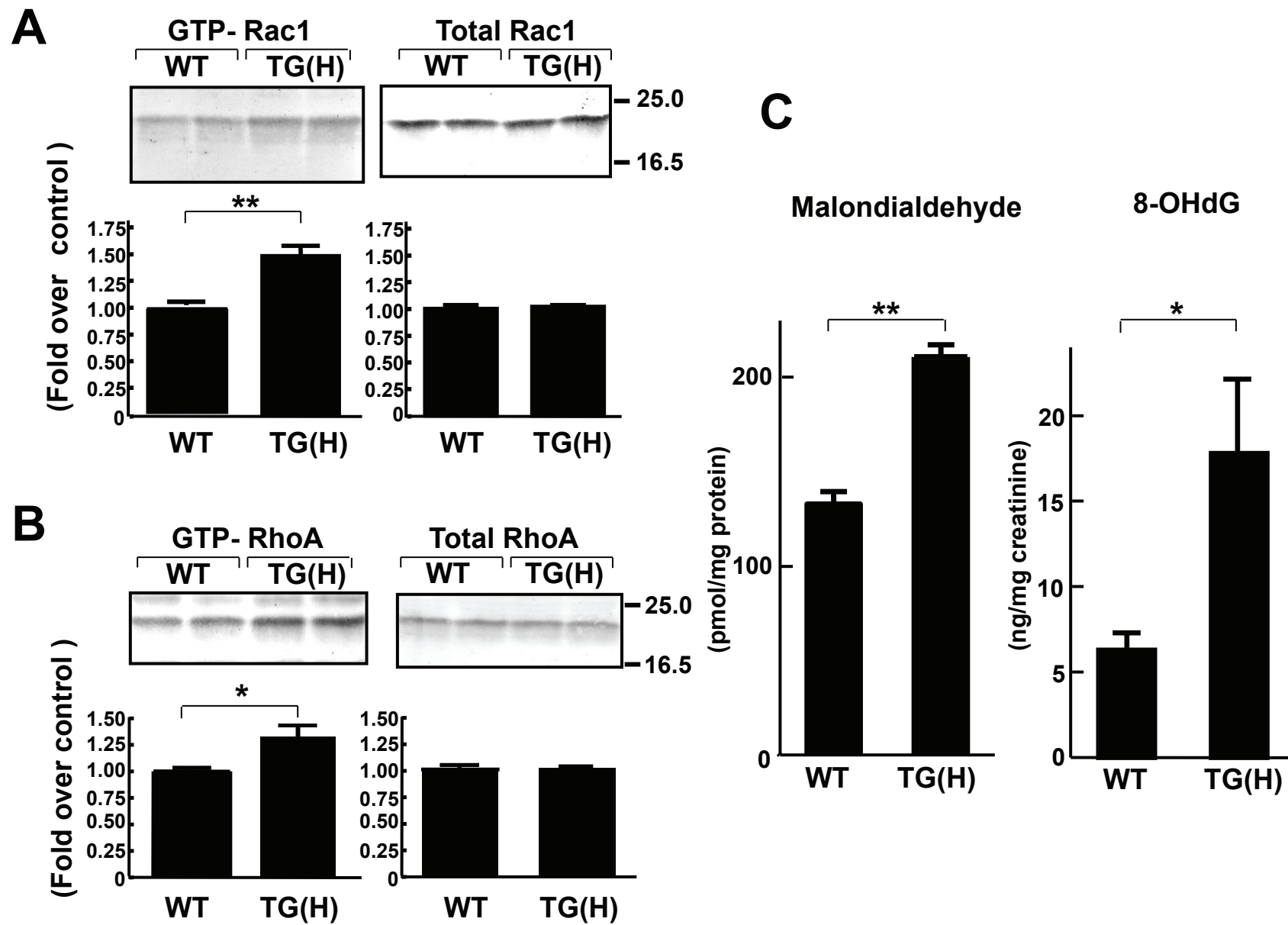
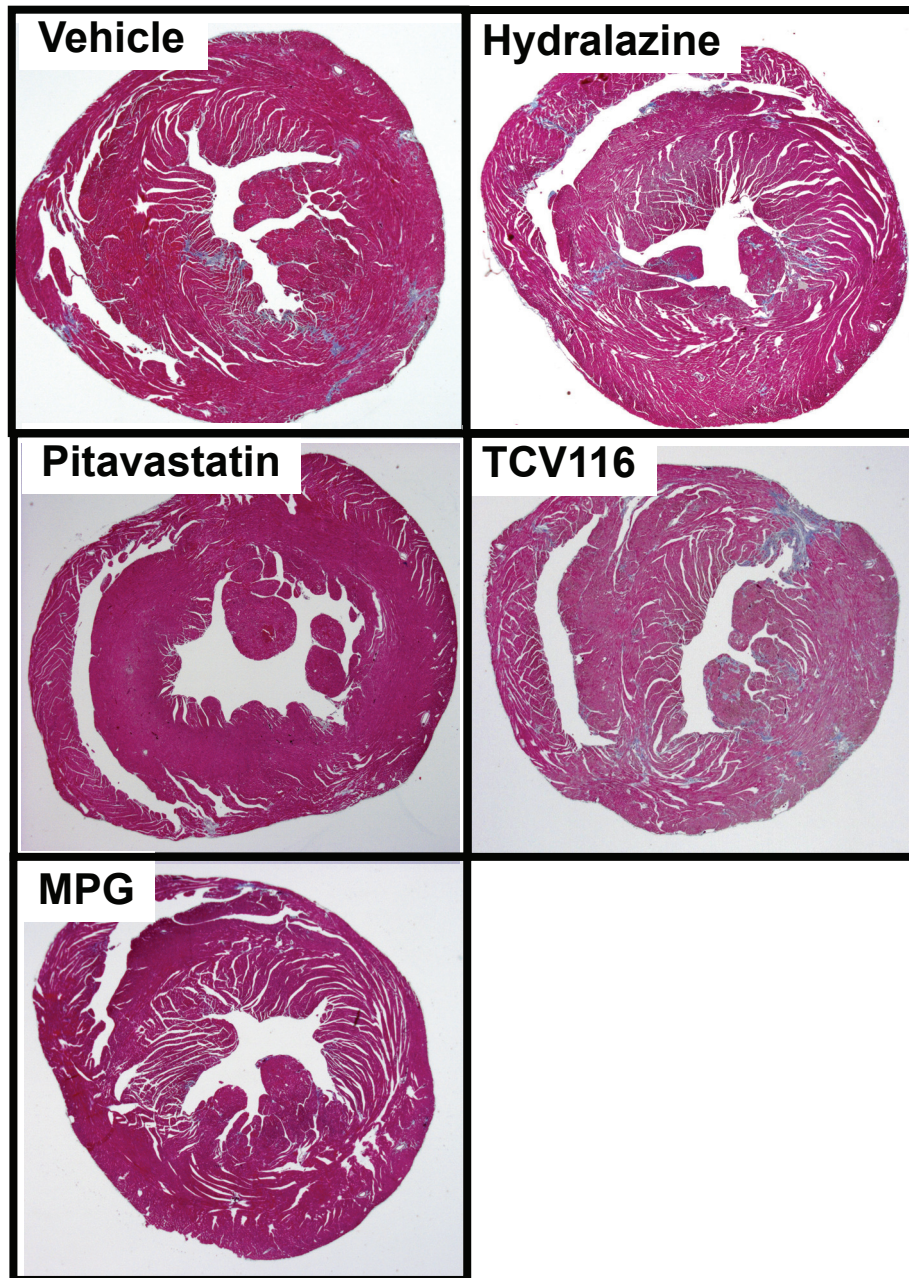
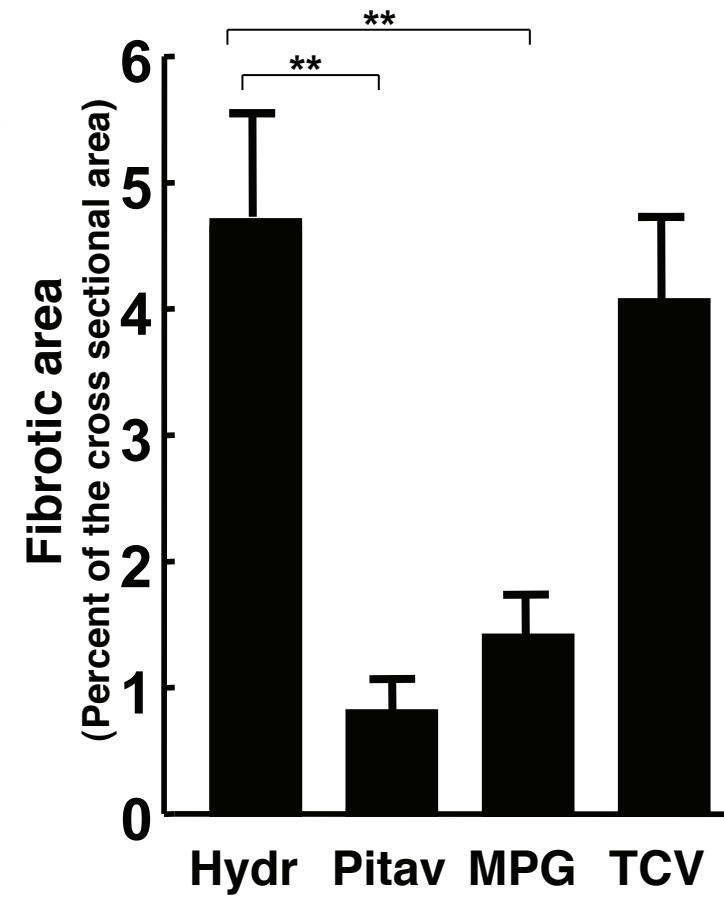


Fig. 4

A**B****Fig. 5**

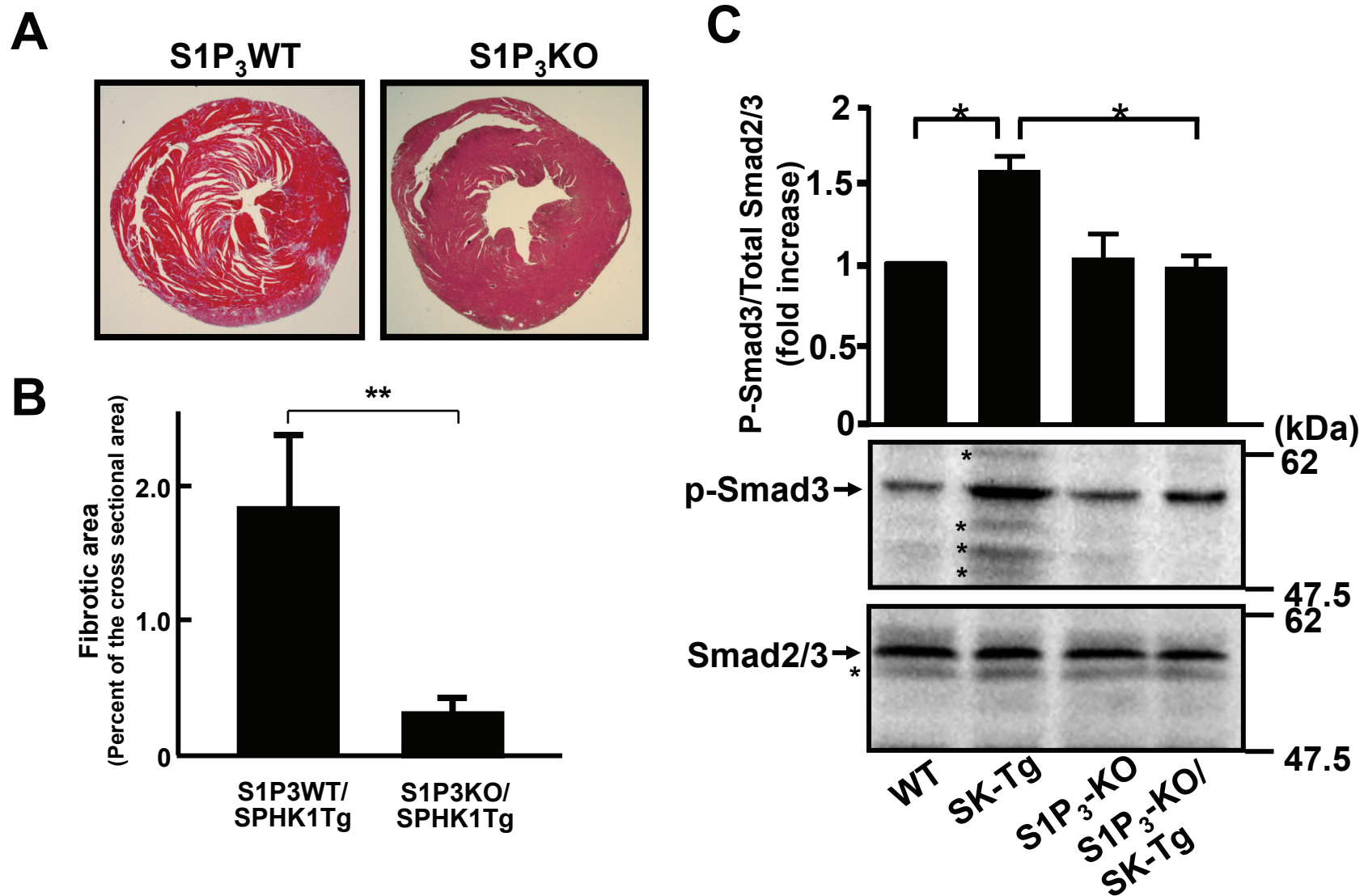


Fig. 6