Supplementary Materials

Materials and Methods

Generation of SPHK1 Transgenic (SPHK1 TG) Mice and $S1P_3$ knock-out (KO)/SPHK1 TG Bigenic Mice

SPHK1 TG mice that overexpres SPHK1a isoform under the universal CAG promoter was generated as described previously[1]. 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'-AGGGGCGTGTTTTCTGTGGAT-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. $S1P_3$ knock-out (KO) mice in C57BL/6 genetic background were described previously[2]. Matings between $S1P_3$ KO and SPHK1 TG mice resulted in generation of $S1P_3$+/-/SPHK1TG and $S1P_3$+/-/WT siblings with the expected 1:1 ratio. $S1P_3$+/-/SPHK1TG and $S1P_3$+/-/WT siblings were then crossed to obtain $S1P_3$+/+/SPHK1TG and $S1P_3$-/-/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 described[3]. The expression level of SPHK1 mRNA was analyzed by Northern blot analysis using a 459 bp *EcoRI-HinCII* fragment of pCAGGS-SPHK1a[1] as a probe as described previously[3]. Probes for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β–myosin heavy chain (β-MHC), skeletal muscle (SKM) α-actin and glyceralaldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control were obtained as described[3]. 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 $S1P_1$, $S1P_2$, $S1P_3$, 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-β1 were 5'-CCAAAGACATCTCACACAGTA-3' and 5'-TGCCGTACAACTCCAGTGAC-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 $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 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 described[7]. 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 α1 and α2 polypeptides, the cardiac homogenates (90 μg protein) prepared by using the buffer A was separated on 8% SDS-PAGE, followed by detection using goat polyclonal anti-type I α1 (#sc-8783) and type I α2 (#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 previously[8]. 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 μg/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 μg) was incubated with 50 μM sphingosine (prepared in mixed micelles in the presence of Triton X-100), 10 μCi of [γ-^{32}P]ATP (1 mM) and 10 mM $MgCl_2$. Labeled lipids were extracted and resolved by thin-layer chromatography as described previously[8].

S1P content in the heart, serum and plasma, and sphingosine content in the heart were quantified as described previously[9]. 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 measured[5].

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 described[10], 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 described[11]. 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 anesthesized 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 depiliated 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 x 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 hervested 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 ventricule, 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 are (infarct area), and total LV area from both sides of each section were measured using Image J (NIH, USA), and each sections were multiplied by the weight of the section and then totaled from all sections. AAR/LV, infarct are/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 previously[7]. 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 previously[14]. In brief, the heart homogenates were prepared in an ice-cold lysis buffer and 10,000 x g supernatant was incubated at 4 ^{o}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 described[13]. 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 ± SEM. ANOVA (analysis of variance) was followed by Dunnette 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

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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 pressue; 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

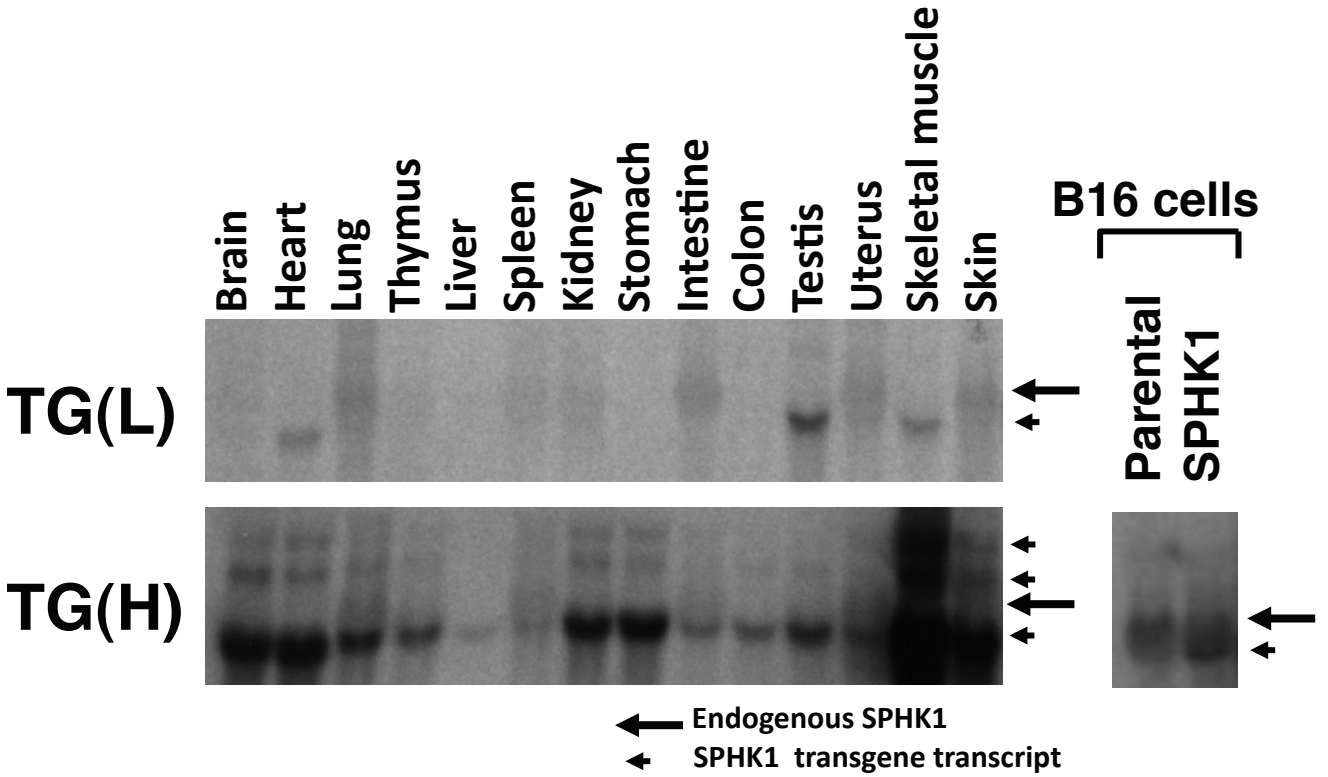
Supplemental Fig. 1. Functional expression of SPHK1a in transgenic mice. (A) Different mRNA expression patterns of the SPHK1a transgene (indicated by *arrowheads*) in two lines of SPHK1aTG mice, TG(L) and TG(H). Total RNA (15 μg) prepared from various organs of TG(L) and TG(H) mice were subjected to 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. 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. 3. 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. 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 α1 and α2 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 α1- and α2-antibodies.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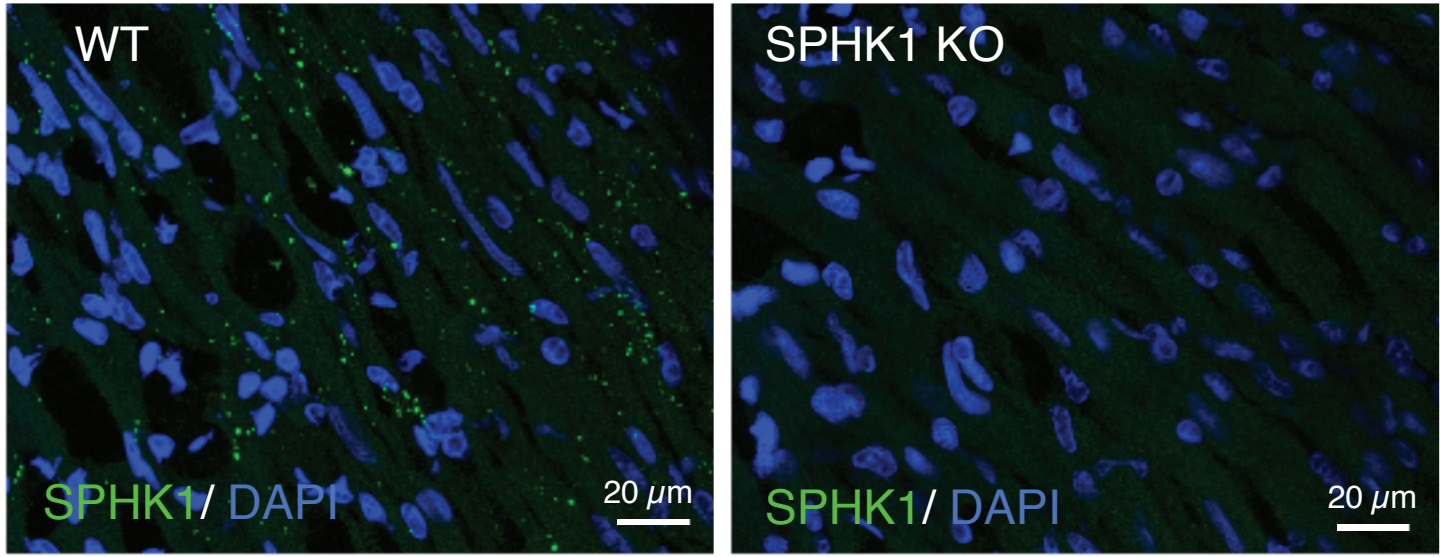
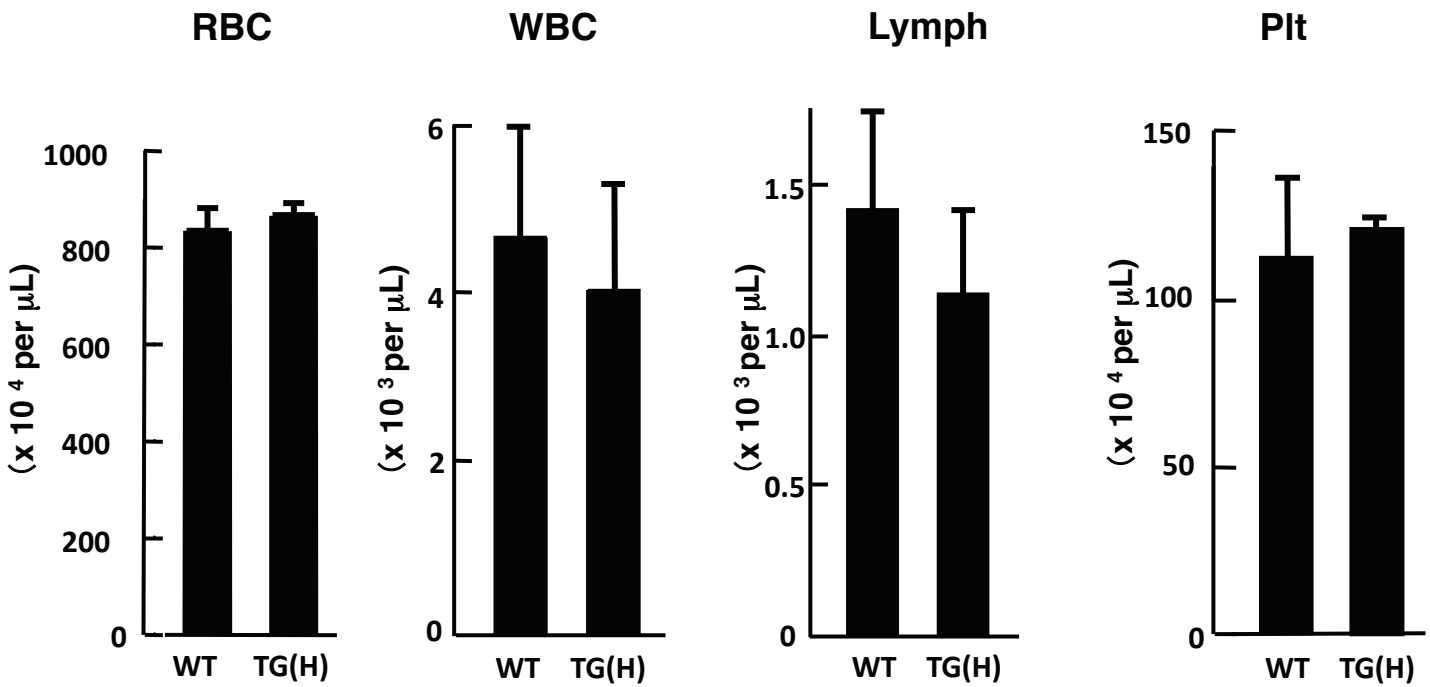
Parental
SPHK1
TG(L)
TG(H)
Endogenous SPHK1
SPHK1 transgene transcript

Supple. Fig. 1



Suppl. Fig. 2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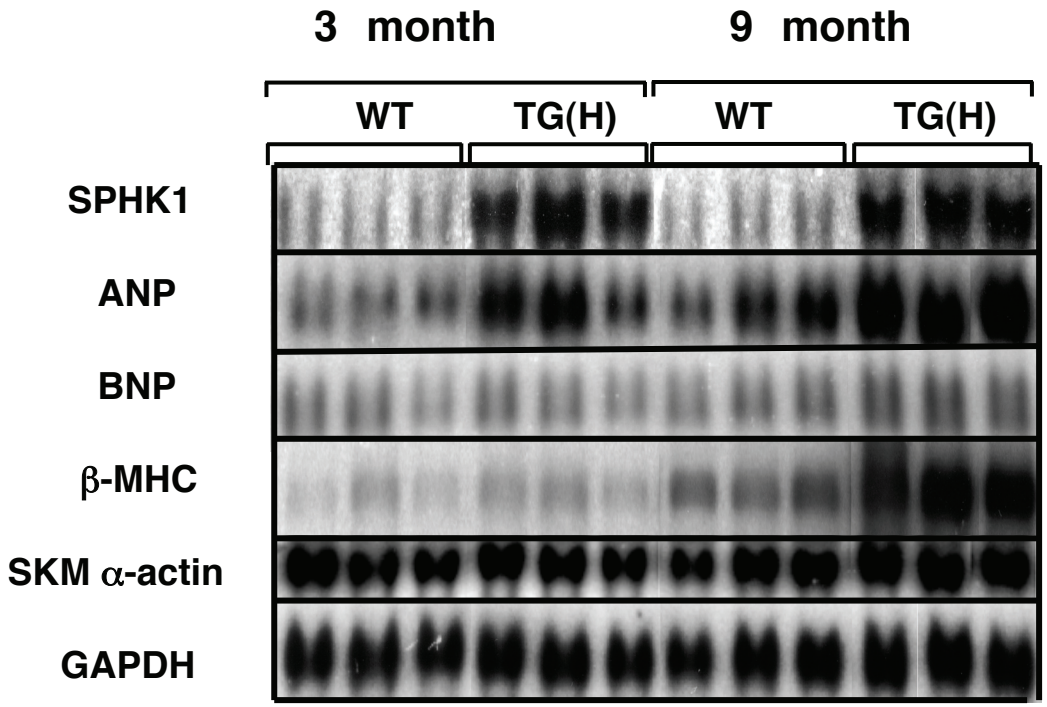
Suppl. Fig. 3


3 month
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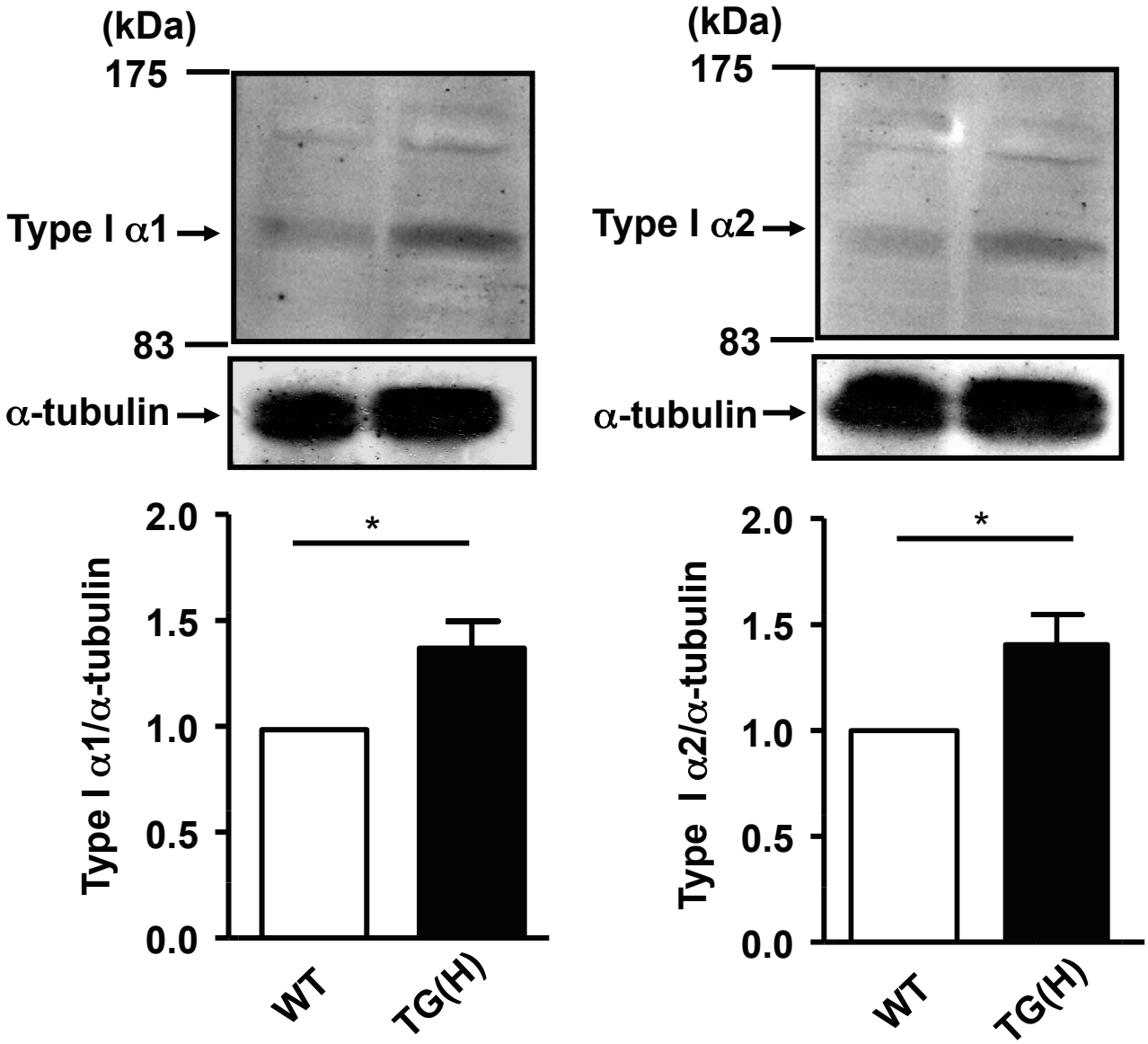
SKM α-actin
GAPDH

Suppl. Fig. 4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Suppl. Fig. 5