SUPPLEMENTARY MATERIALS AND METHODS

Human adipose-derived stromal cells (hAD-SCs)

Utilization of discarded human tissues for research purposes was approved by the Institutional Review Board (IRB) of Asan Medical center (approval number: 2012-0283). Adipose tissue was obtained from young woman undergoing liposuction. It was treated with 0.075 % collagenase type I (Worthington) in phosphate-buffered saline solution (PBS) for 30 min at 37 °C with gently shaking. Collagenase was inactivated by addition of an equal volume of alpha-minimum essential medium (α-MEM, Gibco) supplemented with 10 % fetal bovine serum (FBS, Gibco) and 1 % penicillin and streptomycin solution (Gibco). This suspension was then centrifuged at 3,000 rpm for 10 min to separate the floating adipocytes from the debris. The cells were plated and incubated in culture medium (α-MEM supplemented with 10 % FBS and 1 % penicillin and streptomycin solution). After 48 h, the non-adherent cells were removed and the adherent cells were washed with PBS. Spindle-shaped cells were obtained by day 4 of culture. Subculture was performed when the cells reached 70-80 % confluence. These cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2.

Stable knockdown of SPHK1 and reagents

For stable knockdown of SPHK1, HEK293T cells were transfected with lentiviral packaging vectors as well as SPHK1 shRNA plasmid using electroporation system and after 48 h incubation, SPHK1 shRNA lentiviral particles were obtained. hAD-SCs were infected by adding the lentiviral particles containing SPHK1 shRNA to the culture and then incubated for 48 h. Then, stable clones expressing SPHK1 shRNA were selected by puromycin (2 μg/ml)

for 48 h. The stable expression of SPHK1 shRNA was detected by Western blotting analysis. Control and SPHK1-specific shRNAs (Cat. No. HSH055207-1-LVRU6GP) were acquired from Genecopoeia™. Sphingosine kinase inhibitor (Cat. No. 567731) was obtained from Calbiochem. Fumonisin B_1 (Cat. No. F1147) and sphingosine 1-phosphate (Cat. No. S9666) were purchased from Sigma Aldrich.

BrdU incorporation

Proliferation of hAD-SCs was assayed by measuring DNA synthesis using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche). Cells were seeded in a 96-well plate at a cell density of 1.0 x 10^3 cells per well and incubated with BrdU for 4 h at 37 °C. After removal of BrdU labeling medium, cells were fixed, washed and then quantified for BrdU staining.

Senescence-associated β-galactosidase staining (SA-β-gal staining)

hAD-SCs were plated in 6-well plates at a cell density of 2.0 x 10^4 cells per well and after 72 h, washed twice with 1 x PBS and then incubated for 15 min at room temperature with fixation solution (0.4 % glutaraldehyde in PBS). Subsequently, cells were washed twice with 1 x PBS and incubated for 16 h at 37 °C with staining solution (X-gal, potassium ferrocyanide and potassium ferricyanide in PBS).

Western blotting

Cells were harvested and homogenized in RIPA buffer containing 1 x Halt protease inhibitor cocktail (Thermo Fisher Scientific) for 30 min on ice. Protein concentration in cell lysates was measured using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

The following primary antibodies were used: anti-P-pRb (1:1000), anti-T-pRb (1:1000), anti-P-p53 (1:1000), anti-T-p53 (1:1000), anti-p21 (1:1000), anti-SPHK1 (1:1000) antibodies from Cell Signaling Technology and anti-β-Actin (1:10000) antibody from Sigma Aldrich. Western blots in Fig. 1D, 2C, 3A, 3E and Supplementary Fig. S2D are representative results from three independent experiments.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from hAD-SCs using Trizol (Invitrogen). qRT–PCR was performed on a Bio-Rad (CFX Connect^{TM}Optics Module) using the TOPreal^{TM}qPCR 2x premix (SYBR Green with low ROX) (Enzynomics) according to the manufacturer's instructions. The sequences of all primers used in qRT–PCR are listed in Supplementary Table 1.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for sphingolipids

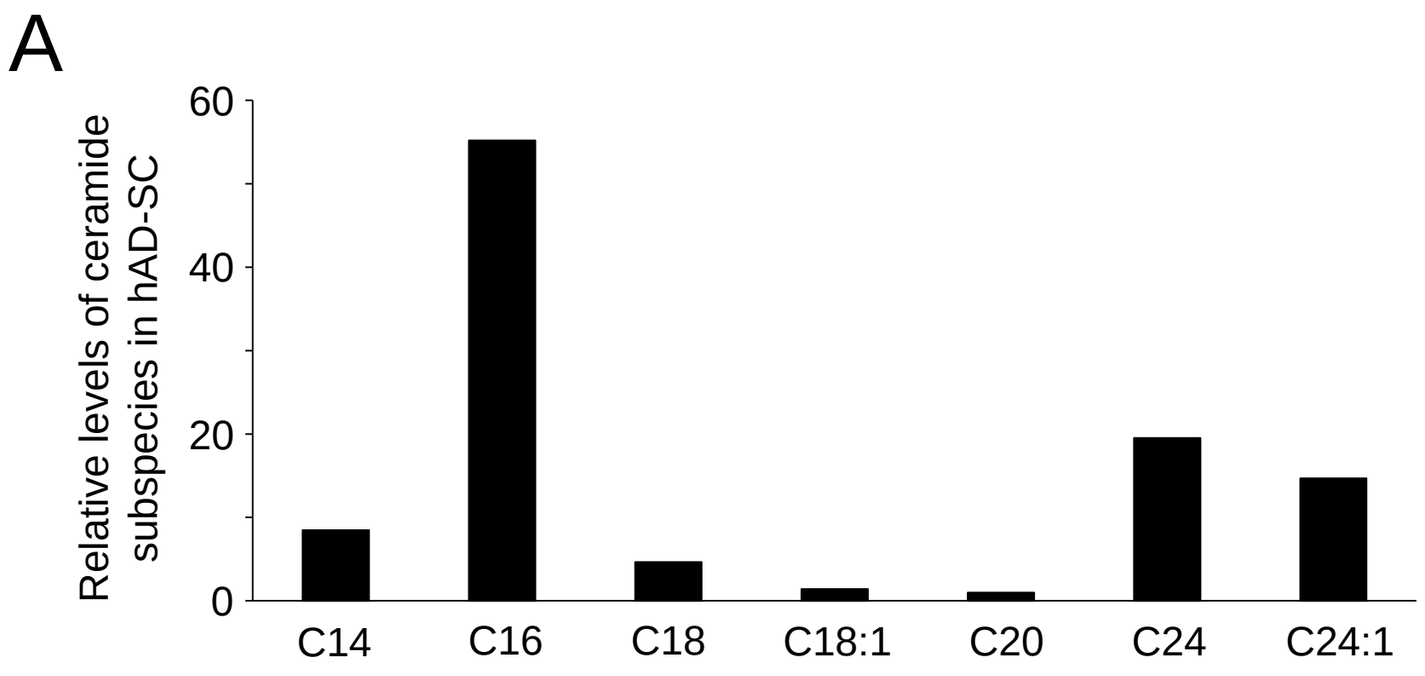
For LC-MS/MS, hAD-SCs were seeded in T175 flasks at a cell density of 4 x 10^5 per flask and incubated for 4 days when the cell density reaches 2.5 x 10^6 per flask. Cells were dissociated using trypsin-EDTA and subsequently subjected to centrifugation, followed by resuspension of the pelleted cells in cold 80 % methanol. These suspended cells were mixed with sphingolipid internal standard solution and centrifuged at 14,000 rpm for 10 min. Chloroform was added to the resultant supernatant and then the mixture was centrifuged at 2000 g for 15 min. Organic layer was collected and subjected to LC-MS/MS analysis.

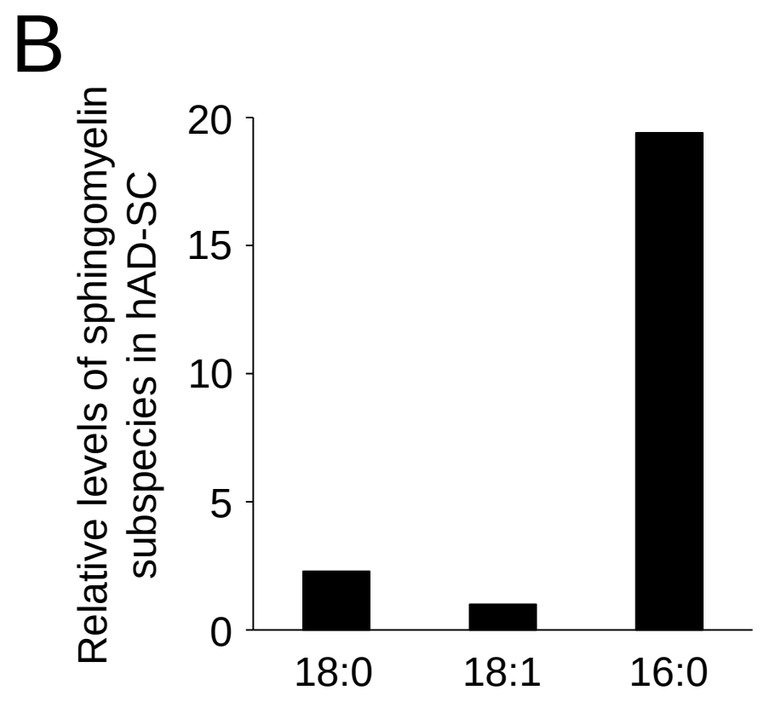
LC-MS/MS system was equipped with Agilent 1290 HPLC (Agilent) and Qtrap 5500 (ABSciex), and reverse phase column (Pursuit 5 C18 150×2.0 mm) was used. Separation gradient for sphingolipids is as follows; mobile phase A (5 mM ammonium formate/MeOH/ tetrahydrofuran, 500/200/300), mobile phase B (5 mM ammonium formate/MeOH/ tetrahydrofuran, 100/200/700), 50 % of A (t = 0 min), 50 % of A (t = 5 min), 30 % of A (t = 8

min), 30 % of A (t = 15 min), 10 % of A (t = 22 min), 10 % of A (t = 25 min), 50 % of A (t = 25.1 min), 50 % of A (t = 30 min) with 200 µl/min at 35 °C. The MRM (multiple reaction monitoring) mode was used in the positive ion mode and the peak area of the extracted ion chromatogram (EIC) corresponding to the specific transition for each lipid was used for quantitation. Data analysis was performed by using Analyst 1.5.2 software. Calibration range for sphingolipids (ceramide, sphingomyelin, sphinganine and sphingosine) was 0.1-1000 nM ($r^2 \geq 0.99$).

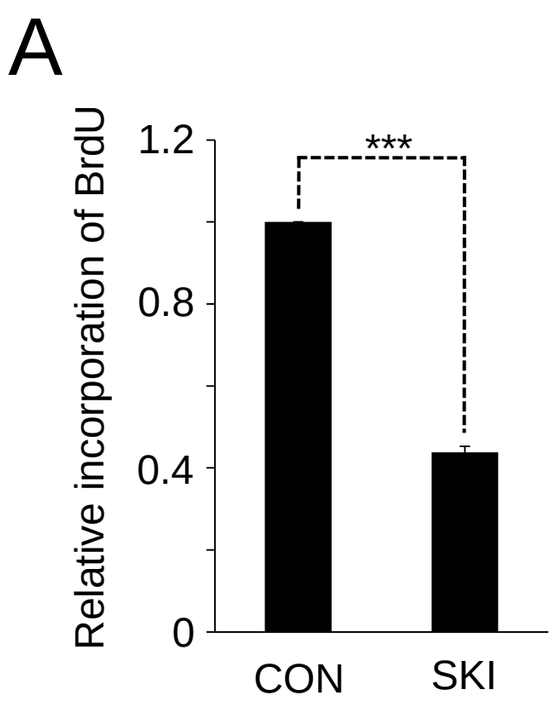
Statistical analysis

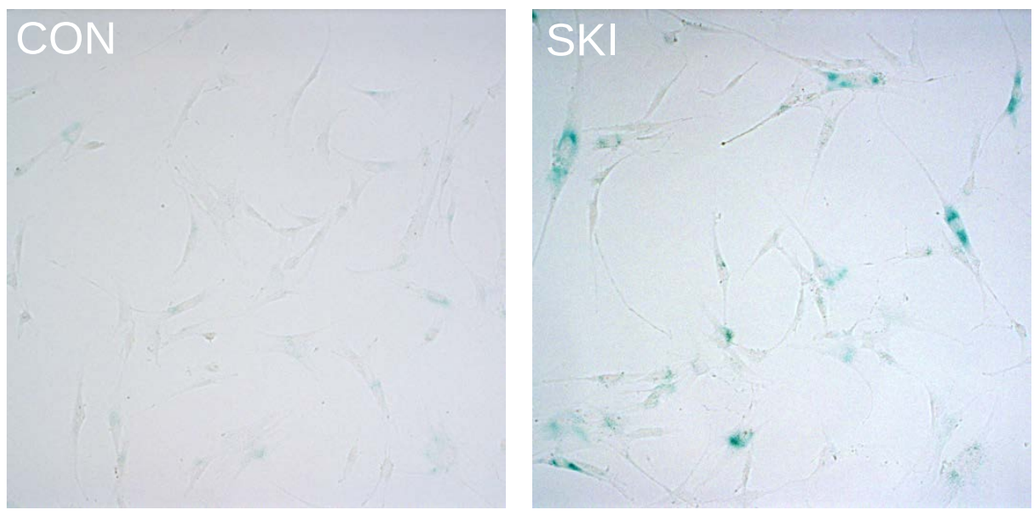
All experimental data were analyzed using the GraphPad Prism 5.01 software program (San Diego, CA, USA) and are expressed as the mean ± standard error (SE) from three independent experiments. $P < 0.05$ was considered statistically significant.

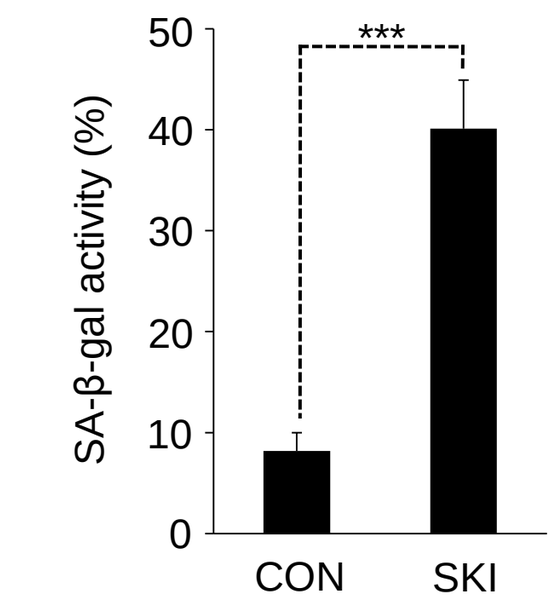


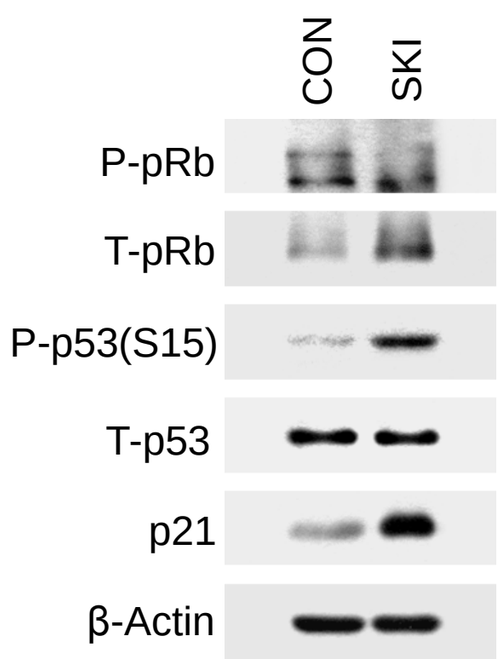


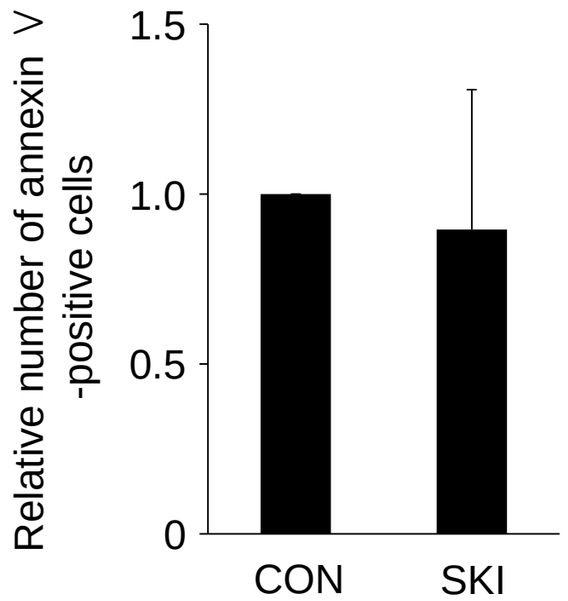
Supplementary Fig. 1. (A, B) LC-MS/MS analysis showing the relative amounts of ceramide (A) and sphingomyelin (B) subspecies in hAD-SCs.



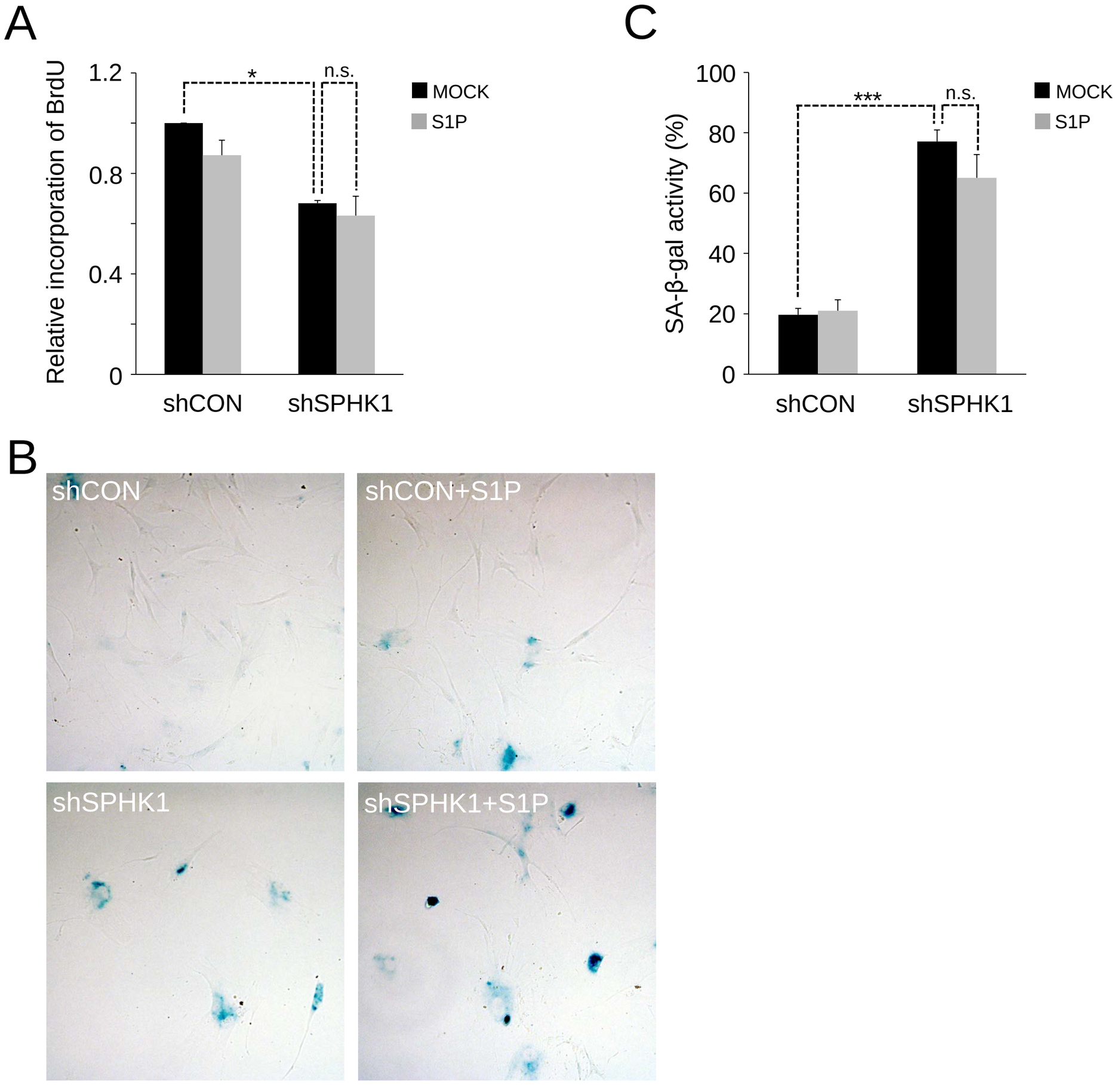




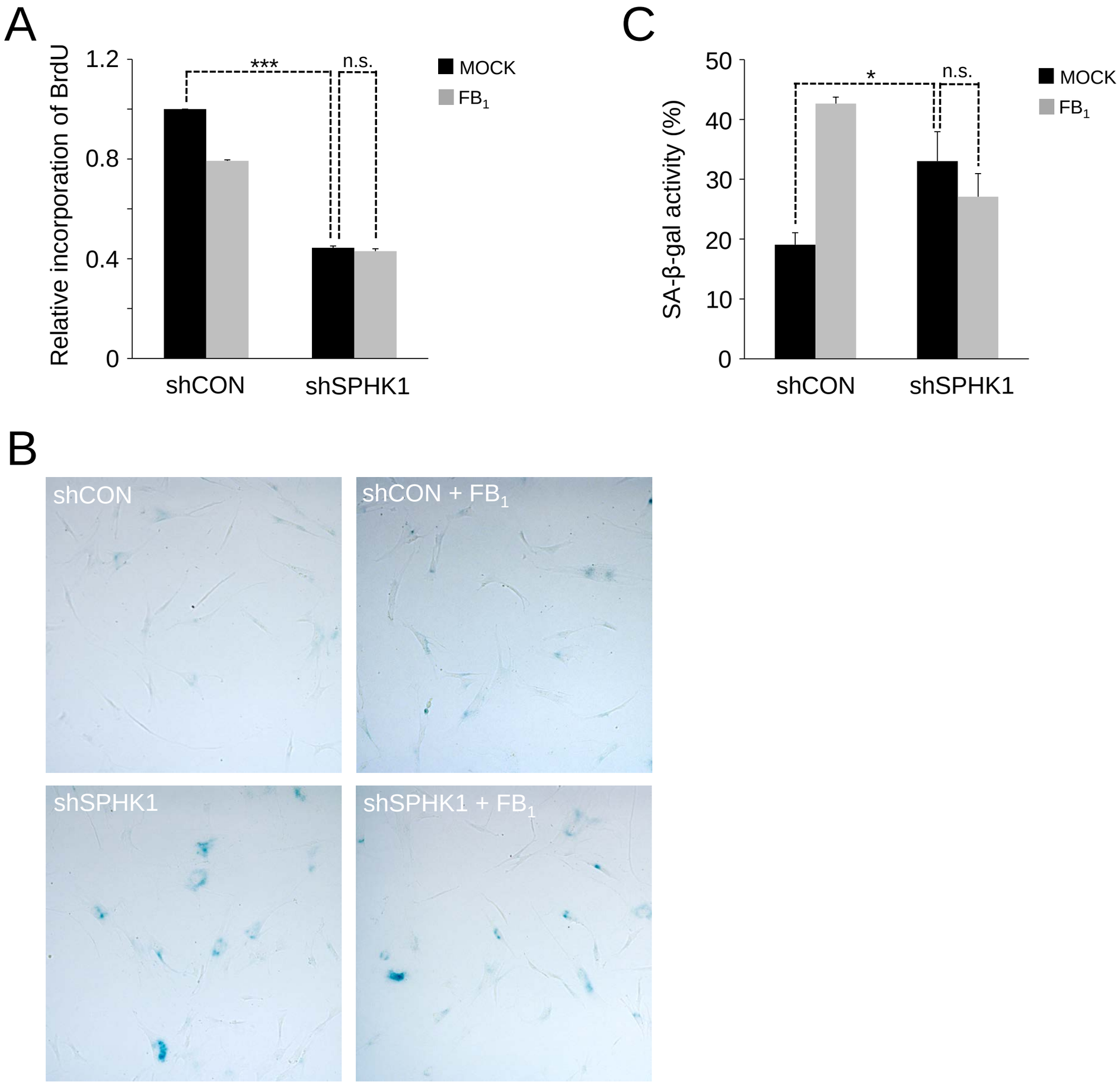




Supplementary Fig. 2. Inhibition of enzymatic activity of SPHK1 accelerates cellular senescence. (A) Treatment with SKI interferes with proliferation of hAD-SCs. ***P<0.001. (B) Activity of SA-β-gal is augmented by treatment with SKI. (C) Quantification of activities of SA-β-gal in DMSO-treated control (CON) and SKI-treated hAD-SCs. ***P<0.001. (D) hAD-SCs treated with SKI exhibit higher expression levels of senescence markers than control cells. (E) SKI-mediated inhibition of SPHK1 activity does not induce apoptosis in hAD-SCs as assayed by annexin Ⅴ staining.



Supplementary Fig. 3. Single supplementation of S1P does not recover senescence enhanced by SPHK1 knockdown. (A) Relative proliferation capacities of shCON or shSPHK1-expressing hAD-SCs which were treated with S1P or not. *P<0.05. (B) Enhanced cellular senescence of SPHK1-silenced cells is not reverted by exogenous addition of S1P as assayed by SA-β-gal activity. (C) Quantification of activities of SA-β-gal in shCON or shSPHK1-expressing cells which were supplemented with S1P or not. ***P<0.001 compared to control shRNA-expressing cells.



Supplementary Fig. 4. Single inhibition of ceramide synthesis does not attenuate senescence accelerated by SPHK1 knockdown. (A) Relative proliferation rates of shCON or shSPHK1-expressing hAD-SCs which were treated with FB_1 or not. (B) Increased activity of SA-β-gal in SPHK1-depleted cells is not reversed significantly by single treatment with FB_1. (C) Quantification of activities of SA-β-gal in shCON or shSPHK1-expressing cells which were treated with FB_1 or not. *P<0.05.

Gene		Primer sequences
SPHK1	F	TCCTGGCACTGCTGCACTC
(Sphingosine kinase 1)	R	TAACCATCAATTCCCCATCCAC
SPHK2	F	AGCAGCAGGACCAGAGGCCA
(Sphingosine kinase 2)	R	GGTGAGGGCAAAGCGTGGG
SMS	F	GAAGCCCAACTGCGAAGAATAA
(Sphingomyelin synthase)	R	AGAGTCGCCGAGGGGAATAC
SMase	F	AAGCCCTGCGCACCCTCAGAA
(Sphingomyelinase)	R	CCTGAAGCTCCCCCACCAGCC
CERS2	F	CCGATTACCTGCTGGAGTCAG
(Ceramide synthase 2)	R	GGCGAAGACGATGAAGATGTTG
CERS5	F	GTTTCGCCATCGGAGGAATC
(Ceramide synthase 5)	R	GCCAGCACTGTCGGATGTC
CERS6	F	GGGATCTTAGCCTGGTTCTGG
(Ceramide synthase 6)	R	GCCTCCTCCGTGTTCTTCAG
CDase	F	GATATTGGCCCCAGCCTACTTT
(Ceramidase)	R	ACCCTGCTTAGCATCGAGTTCA
SPT	F	GGTGGAGATGGTACAGGCG
(Serine palmitoyltransferase)	R	TGGTTGCCACTCTTCAATCAG
SPL	F	TGGAGGTGGATGTGCGGGCAA
(Sphingosine-1-phosphate lyase)	R	CCCAGACAAGCGTCGACATGAAG
S1PP	F	CCATTTGTGGACCTGATTGACA
(Sphingosine-1-phosphate phosphatase)	R	ACTTCCTAGTATCTCGGCTGTG
GAPDH	F	TGAACGGGAAGCTCACTG
	R	TCCACCACCCTGTTGCTGTA

Supplementary Table 1. qRT-PCR primer sequences