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Corresponding Author: Sun-Cheol Choi

Authors: Min Kyung Kim[1], Wooseong Lee[1], Gang-Ho Yoon[2], Eun-Ju Chang[1,2], Sun-Cheol Choi[1,2,*], Seong Who Kim[1]

Institution: [1]Department of Biochemistry and Molecular Biology and [2]Department of Biomedical Sciences, University of Ulsan College of Medicine,

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Links between accelerated replicative cellular senescence and down-regulation of SPHK1 transcription

Min Kyung Kim[1], Wooseong Lee[1], Gang-Ho Yoon[2], Eun-Ju Chang[1, 2], Sun-Cheol Choi[1, 2,*] and Seong Who Kim[1,*]

[1]Department of Biochemistry and Molecular Biology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, 05505 Republic of Korea

[2]Department of Biomedical Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul, 05505 Republic of Korea

*Corresponding authors

Running title: Role of SPHK1 in cellular senescence

Tel: 82-2-3010-2206

Fax: 82-2-3010-5307

E-mail: choisc@amc.seoul.kr, swhokim@amc.seoul.kr

ABSTRACT

We have identified a mechanism to diminish proliferation capacity of cells during cell expansion by using human adipose-derived stromalcells (hAD-SCs) as a model of replicative senescence. hAD-SCs of high passage numbers exhibited a reduced proliferation capacity with accelerated cellular senescence. The levels of key bioactive sphingolipids were significantly increased in these senescent hAD-SCs. Notably, transcription of sphingosine kinase 1 (SPHK1) was down-regulated in hAD-SCs at high passage numbers. SPHK1 knockdown as well as inhibition of its enzymatic activity impeded proliferation of hAD-SCs, with concomitant induction of cellular senescence and accumulation of sphingolipids as in high passage cells. SPHK1 knockdown-accelerated cellular senescence was attenuated by co-treatment of sphingosine-1-phosphate and an inhibitor of ceramide synthesis, fumonisin B_1 but not by treatment of either one. Together, these results suggest that down-regulated transcription of SPHK1 is a critical inducer of altered sphingolipid profiles, enhancing replicative senescence during multiple rounds of cell division.

Keywords: Sphingolipid, Sphingosine kinase 1, SPHK1 transcription, Replicative senescence, Human adipose-derived stromal cells

INTRODUCTION

Sphingolipids are essential components of eukaryotic cells and exist in the form of some metabolites including sphingomyelins, ceramides and sphingosine. Besides playing structural roles in cell membrane, they function as bioactive signaling molecules to regulate cell proliferation, differentiation, migration, and apoptosis (1, 2). Sphingolipid metabolic pathways exhibit an intricate network of reactions involving a variety of enzymes. In this pathway, sphingosine is a backbone of all sphingolipids. Sphingomyelins are produced by sphingomyelin synthases using phosphatidylcholine and ceramide (3). Ceramide, a central sphingolipid, is synthesized by the *de novo* pathway and the salvage pathway through the action of serine palmitoyltransferase and ceramide synthase, respectively (4). Sphingosine kinase (SPHK) 1 and 2, major enzymes in sphingolipid metabolism, catalyze the phosphorylation of sphingosine to generate sphingosine-1-phosphate (S1P). S1P acts as a bioactive molecule to bind to five specific G-protein-coupled receptors (S1P receptors), thus regulating various intracellular signaling pathways during cell proliferation and migration (5-8).

Several pieces of evidence have demonstrated that sphingolipids play crucial roles in regulation of lifespan in model organisms such as worms, flies and yeast (8). These lipid species have also emerged as critical regulators of replicative senescence at the cellular level. Replicative cellular senescence causes proliferation capacity of cells to become progressively diminished during their repeated replication (9, 10). At this senescent stage, cells lose their replicative potential, leading to gradual impairment of their physiological functions (11). In addition, senescent cells are resistant to diverse stimuli such as mitogenic and apoptotic ones, though they are still viable and metabolically active (12). Generally, senescence triggers morphological changes such as enlarged and flattened cellular shapes and induces a tumor

suppressor network involving the ARF/p53 and p16/pRb pathways, which is used as biomarkers to identify the senescent cells (13-15). ARF promotes the stabilization of p53 protein followed by increased expression of p21, thereby contributing to the activation of the p16/pRb pathway. Thus, the interaction between these two tumor suppressor pathways maintains the senescent state of cells (16-18).

Human adipose-derived stromal cells (hAD-SCs) have been proposed as the attractive cell types for cell-based therapies and regenerative medicine. However, hAD-SCs have a drawback related to *in vitro* cell expansion that is the difficulty of generating sufficient numbers of cells for repeated clinical applications, possibly arising from replicative senescence that occurs during continuous cell expansion. To circumvent this cell expansion limitation, we sought to elucidate the molecular mechanisms underlying the diminished proliferation capacity of hAD-SCs. Recent studies have revealed the implication of ceramides or sphingosine in cellular senescence (8). SPHKs have also been shown to contribute to cell survival, proliferation and resistance to apoptosis (19, 20), though their roles in cellular senescence remain to be investigated. In this study, we have found that alteration of sphingolipid profiles occurs during senescence of hAD-SCs. Notably, both these changes in the levels of sphingolipids and cellular senescence are caused by down-regulated transcription of SPHK1, which is entailed by repeated cell-cycle progression.

RESULTS

Continuous expansion of hAD-SCs induces cellular senescence and accumulation of sphingolipids

To identify the precise molecular mechanisms by which proliferation capacity of hAD-SCs is reduced during *in vitro* cell expansion, we first checked the proliferation rates of these cells undergoing repeated cell division. To this end, we used BrdU incorporation to assay DNA synthesis/cell cycle progression in early (3 or 4 passages)- and late (17 or 18 passages)-passage cells. As expected, hAD-SCs of high passage numbers had a markedly diminished proliferation rate as demonstrated by incorporation of less BrdU into late-passage cells than early-passage cells (Fig. 1A). In line with this, the late-passage cells exhibited a higher activity of senescence-associated-β-galactosidase (SA-β-gal) than the early-passage cells (Fig. 1B, C). Consistently, the levels of cellular senescence markers, including phospho-p53 and p21, were highly enhanced in the late cells, compared to those in the early-passage cells (Fig. 1D). In contrast, the level of phosphorylated pRb, which allows cell cycle progression, was lower in the late-passage cells than in the early cells (Fig. 1D). Together, these results indicate that hAD-SCs undergo cellular senescence during continuous *in vitro* expansion.

Accumulating evidence reveals critical roles of bioactive sphingolipids in mechanism of mammalian cell senescence (8). Thus, we hypothesized that these lipid metabolites could function as potential mediators of hAD-SCs senescence. To test this assumption, we first used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the most abundant subspecies of ceramide and sphingomyelin in hAD-SCs. As shown in Supplementary Fig. S1, C16 ceramide and 16:0 sphingomyelin were found to be the major subspecies in these cells. We next examined whether changes in the cellular levels of sphingolipids including these main subspecies would occur in hAD-SCs, depending on their passage numbers. Importantly, the levels of key sphingolipid species, C16 ceramide, 16:0 sphingomyelin, sphinganine and sphingosine, were significantly higher in the late-passage cells than those in the early-passage cells (Fig. 1E). Taken together, these results suggest that

multiple rounds of cell division induce accumulation of anti-growth bioactive sphingolipids, such as ceramide and sphingosine, thereby resulting in replicative senescence in hAD-SCs.

Reduced transcription of SPHK1 in senescent hAD-SCs

The alteration of sphingolipid profiles as shown above indicates the possibility that the expression and/or activity of sphingolipid metabolic enzymes could be changed in senescent hAD-SCs. We next compared the expression levels of several sphingolipid metabolic enzymes in the late-passage cells with those in the early-passage cells. Of note, quantitative RT-PCR analysis revealed that higher passage hAD-SCs have a significantly reduced level of sphingosin kinase 1 (SPHK1) mRNA, compared to that in lower passage ones (Fig. 2B). In contrast, neither SPHK2, an isoform of SPHK1 nor the other key sphingolipid metabolic genes exhibited a marked change in the amount of each transcript as the passage number increases. Consistent with the lowered level of its mRNA, the expression of SPHK1 protein was also highly decreased in the late-passage cells, compared to that in the early-passage cells (Fig. 2C, D). Therefore, these results suggest that transcription of SPHK1 is down-regulated during hAD-SCs senescence.

Silencing of SPHK1 leads to cellular senescence and accumulation of sphingolipids

Given that SPHK1 is a key regulator of the balance of anti-growth sphingolipids, ceramide and sphingosine and pro-growth sphingolipid, S1P (21), it is possible that down-regulation of SPHK1 transcription might be a critical inducer of the accumulation of sphingolipids and cellular senescence in high passage hAD-SCs. To test this possibility, we carried out lentiviral

shRNA-mediated silencing of SPHK1 or repressed its enzymatic activity to examine sphingolipid profile and cellular senescence in cells lacking the action of SPHK1. We tested the efficacies of three shRNAs specific for SPHK1 (designated shSPHK1 #1, #2 or #3) by Western blot analysis. Although all three shRNAs silenced SPHK1 (Fig. 3A), we used shSPHK1 #2 for further experiments because it is more effective, compared to the other two shRNAs. Knockdown of SPHK1 as well as treatment with SKI, a SPHK1 inhibitor reduced significantly incorporation of BrdU into cellular DNA, compared to that in control cells (Fig. 3B; Supplementary Fig. S2A), indicative of the lowered proliferation ability. Like high passage cells, SPHK1-depleted or SKI-treated hAD-SCs underwent accelerated cellular senescence as evidenced by higher activity of SA-β-gal (Fig. 3C, D; Supplementary Fig. S2B, C). In support of this, the levels of cellular senescence markers, phospho-p53 and p21 were up-regulated in the SPHK1-silenced or SKI-treated cells with the level of phospho-pRb, a marker of cell proliferation reduced (Fig. 3E; Supplementary Fig. S2D). Furthermore, LC-MS/MS analysis revealed elevated levels of key sphingolipids, including ceramide and sphingosine, in SPHK1-depleted cells compared to those in negative control shRNA-expressing cells (Fig. 3F). siRNA-mediated silencing of SPHK1 has been shown to induce apoptosis in MCF-7 breast cancer cells (22). Thus, we also checked whether treatment with SKI could induce this programmed cell death in hAD-SCs. In contrast, annexin V staining revealed no significant increase in the number of apoptotic cells in SKI-treated hAD-SCs (Supplementary Fig. S2E). Thus, it seems likely that down-regulation of SPHK1 expression or activity induces cellular senescence and/or apoptosis depending on the cell types and cell-context. Overall, these data support that reduced transcription of SPHK1 is responsible for altered sphingolipid profiles and cellular senescence which are elicited by continuous cell expansion.

Alteration of sphingolipid profiles causes cellular senescence in SPHK1-depleted cells

While ceramide and sphingosine are usually implicated in induction of cell-cycle arrest, apoptosis, and senescence, sphingosine-1-phosphate (S1P) promotes cell proliferation, survival, and migration (23). Thus, we speculated that cellular senescence accelerated by SPHK1 knockdown might be due to increased ceramide levels and/or decreased S1P levels. To test this hypothesis, we investigated whether SPHK1 knockdown-induced cellular senescence could be attenuated by inhibition of ceramide synthesis or exogenous supplementation of S1P during cell culture. BrdU incorporation assays showed that the proliferation impeded by shRNA-mediated silencing of SPHK1 could not be recovered by single treatment with S1P or fumonisin B_1 (FB_1), a ceramide synthesis inhibitor and even by co-treatment with both (Fig. 4A; Supplementary Fig. S3A, S4A), indicating the irreversibility of cellular senescence. In contrast, the elevated activity of SA-β-gal in SPHK1-silenced cells could be reduced by simultaneous co-treatment with S1P and FB_1 but not by single treatment with either one (Fig. 4B, C; Supplementary Fig. S3B, C and S4B, C), suggesting that cellular senescence accelerated by SPHK1 depletion may result from increase in ceramide levels with concurrent decrease in S1P levels. Taking together, we conclude that depletion of SPHK1 alters the balance of pro-death and anti-death bioactive sphingolipids, enhancing replicative cellular senescence.

DISCUSSION

Recent studies have demonstrated that sphingolipids, such as ceramide and sphingosine, and their metabolic enzymes are involved in regulation of cellular senescence in various cell

types (8). However, it remains elusive how changes in the levels of sphingolipid species and in the activities of their metabolic enzymes are induced in cells undergoing replicative senescence. In this study, we have shown that down-regulation of SPHK1 expression at the transcriptional level acts as a key inducer of altered sphingolipid profiles, thereby accelerating cellular senescence during cell expansion. Like most of human cell types, human adipose tissue-derived stromal cells (hAD-SCs), a model of replicative senescence used in this work, exhibited a decrease in proliferation capacity with concomitant enhancement of cellular senescence as they undergo increasing numbers of passages. Previously, human senescent fibroblasts of high passage numbers were shown to have increased ceramide levels and sphingomyelinase (SMase) activity, compared to ones of lower passage numbers (5). It is of note that the senescent hAD-SCs of high passage numbers have significantly elevated levels not only of ceramide but of other sphingolipids including sphingomyelin, sphingosine and sphinganine (Fig. 1). In addition, among several sphingolipid metabolic enzymes, expression of SPHK1 only was significantly down-regulated at the mRNA level as well as the protein level in the senescent hAD-SCs. In support of pivotal role for this decreased expression of SPHK1 in inducing senescence, shRNA-mediated silencing of SPHK1 promoted cellular senescence at the expense of proliferation and up-regulated the levels of the same sphingolipid species as observed in late-passage cells. Pharmacological inhibition of SPHK1 activity also suppressed cell growth but accelerated cellular senescence. Furthermore, the enhanced cellular senescence in SPHK1-depleted cells could be attenuated by exogenous addition of S1P with concurrent inhibition of ceramide synthesis. Given these findings, it is possible that reduced transcription of SPHK1 may function as a bona fide inducer of replicative senescence by regulating sphingolipid metabolism during repeated rounds of cell division.

Cellular levels of SPHK1 expression have been shown to play key roles in sphingolipid-mediated determination of cell fates. The levels of SPHK1 mRNA are generally increased in various types of human cancers (24). In line with this, overexpression of SPHK1 promotes focus formation, growth of cells in soft agar and tumor formation in NOD/SCID mice (25), pointing to its oncogenic role but reduces cellular apoptosis (26). Increased levels of S1P by overexpression of SPHK1 also expedite cell-cycle progression and enhance cell growth, whereas overexpression of S1P phosphatase increases cell death (27). Thus, it appears that up-regulation of SPHK1 expression leads to pro-growth and anti-apoptotic signals mediated by the SPHK1/S1P pathway. Down-regulation of SPHK1 expression provides a mechanism of tumor suppression by modulating the levels of sphingolipid species as evidenced by analysis of double-KO mice lacking a tumor suppressor p53 and SPHK1. p53-null mice develop thymic lymphomas, which were completely abrogated by deletion of SPHK1 in these mice (28). In p53-KO mice, expression of SPHK1 and levels of S1P are elevated, whereas levels of ceramide are decreased, compared to wild-type mice. These sphingolipid profiles in p53-null mice are reversed by deletion of SPHK1, in turn accompanied by cellular senescence but not by apoptosis. Notably, SPHK1 has been shown to undergo proteolytic degradation in response to genotoxic stress in a p53-dependent manner (29). These results suggest that p53 induces its tumor suppressor effects by down-regulating expression of SPHK1 at the posttranslational level, leading to decreased levels of S1P and increased levels of ceramide and sphingosine, which then mediate cellular senescence. As shown in our results, down-regulation of SPHK1 expression at the transcriptional level causes similarly elevation of ceramide and sphingosine levels, contributing to replicative cellular senescence. Given these results, it is tempting to speculate that mode of regulation of SPHK1 expression to induce cellular senescence depends on upstream inducers and physiological context. It

seems likely that for tissue homeostasis, a rapid response mechanism for inducing senescence, such as p53-mediated proteolysis of SPHK1, is necessary for cells to prevent immediately tumorigenesis in response to genotoxic stresses including DNA damage-inducing agents and oncogenic activation. In contrast, a relatively slower mechanism, such as down-regulation of SPHK1 transcription, may be appropriate for inducing replicative senescence in response to telomere shortening and oxidative stress entailed by multiple rounds of cell division. However, it cannot be excluded that both the rapid and slow mechanisms might function in parallel for preventing tumor formation and/or inducing replicative senescence. A few modes of regulation would ensure a robust mechanism to induce cellular senescence in the right place at the right time during tissue homeostasis and aging.

As sphingolipid species, including sphingomyelin, ceramide, sphingosine and S1P, are interconvertible, knockdown of one sphingolipid metabolic enzyme would alter relative levels of these metabolites. Furthermore, the levels of activity of a sphingolipid metabolic enzyme could affect those of other enzymes. Overexpression of S1P phosphatase-1, which dephosphorylates S1P, enhances *de novo* synthesis of ceramide, possibly owing to de-repression of serine palmitoyltransferase or ceramide synthase by S1P (21). Treatment with fumonisin B_1, an inhibitor of ceramide synthase, has been shown to increase the activity of acidic sphingomyelinase, accompanied with a decrease of sphingomyelin, and the expression and activities of serine palmitoyltransferase and SPHK1 in mouse liver (30). Thus, 'sphingolipid rheostat' has been proposed to regulate relative levels of sphingolipid metabolites, thereby determining cell fates (21). SPHK1 acts as a critical regulator of sphingolipid rheostat to maintain a balance between pro-growth and pro-death bioactive sphingolipids. As shown by our assays, knockdown of SPHK1 seemed to tilt the balance of ceramide, sphingosine and S1P in favor of growth inhibition and cell death. SPHK1

knockdown-reduced proliferation capacity could be recovered by neither co-treatment withboth S1P and a ceramide synthesis inhibitor, fumonisin B_1 nor single treatment with either one, indicative of the irreversibility of cellular senescence accelerated by depletion of SPHK1. However, this cellular senescence could be attenuated efficiently by co-treatment with both the reagents but not by treatment with either one, suggesting the crucial roles that increased ceramide levels and decreased S1P levels each play in promoting replicative senescence in SPHK1-depeleted cells. In most cell types, the cellular levels of ceramide, sphingosine and S1P differ approximately by an order of magnitude, with ceramide being present at the highest and S1P at the lowest level (2). A small change in ceramide can lead to profound changes in S1P. In addition, the levels of ceramide in SPHK1-depeleted cells are about two times higher than those in normal control cells (Fig. 3). Despite these facts, inhibition of ceramide generation alone was unable to attenuate acceleration of cellular senescence by knockdown of SPHK1, thus revealing the strong potency of low levels of S1P in shifting the balance between pro- and anti-death signals toward cell survival.

In conclusion, this study has found that down-regulation of SPHK1 transcription serve as a critical inducer of a tilt in the balance between pro- and anti-growth bioactive sphingolipids in favor of growth inhibition and cellular senescence during *in vitro* cell expansion. Further studies are warranted to elucidate the molecular mechanisms by which SPHK1 expression is regulated negatively at the transcriptional level during multiple rounds of cell divisions. Since SPHK1 is an essential regulator of sphingolipid rheostat, better understanding of control of its expression holds promise to develop novel tools to prolong the proliferation capacity of cells for cell-based therapy and regenerative medicine.

MATERIALS AND METHODS

Detailed information can be found in the Supplementary data.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

FIGURE LEGENDS

Fig. 1. High passage hAD-SCs undergo cellular senescence and changes in sphingolipid profile. (A) BrdU incorporation assay showing reduced proliferation capacity of late-passage cells relative to early-passage cells. Error bars indicate the standard error (SE). Asterisks above the bars denote ***P<0.001 compared to early-passage cells. (B) Late-passage hAD-SCs display higher activity of senescence-associated-β-galactosidase (SA-β-gal) than early-passage cells. (C) Quantification of activities of SA-β-gal in early- and late-passage hAD-SCs. For this, the number of SA-β-gal positive cells per field was counted. ***P<0.001 (D) Cellular levels of senescence markers, phospho-p53 and p21 are increased in late-passage cells, compared to those in early-passage cells, as analyzed by Western blotting. β-Actin serves as a loading control. (E) Elevation of levels of sphingolipid species in senescent late-passage hAD-SCs. Cer, ceramide; SM, sphingomyelin; SA, sphinganine; SO, sphingosine. *P<0.05.

Fig. 2. Transcription of SPHK1 is reduced in senescent hAD-SCs. (A) Scheme of sphingolipid metabolism. SPT, serine palmitoyltransferase; SMase, sphingomyelinase; SMS, sphingomyelin synthase; CDase, ceramidase; CERS, ceramide synthase; SPHK1, sphingosine kinase 1; S1PP, sphingosine-1-phosphate phosphatase; SPL, sphingosine-1-phosphate lyase. (B) Late-passage hAD-SCs have lower levels of SPHK1 mRNA than early-passage cells as shown by qRT-PCR. **P<0.01 compared to early-passage cells. n.s. not significant. (C) Relative expression of SPHK1 protein in early- and late-passage cells. β-Actin is a loading control. (D) Quantification of SPHK1 protein levels in (C) (normalized to β-Actin). *P<0.05.

Fig. 3. Knockdown of SPHK1 promotes cellular senescence and accumulation of

sphingolipids. (A) Western blotting showing knockdown efficacies of shRNAs against SPHK1 (shSPHK1 #1-3). shCON, a negative control shRNA. (B) Proliferation capacity is decreased in SPHK1-depleted hAD-SCs. **P<0.01 compared to control shRNA-expressing cells. (C) Cellular senescence is enhanced in SPHK1-silenced hAD-SCs as shown by relatively higher activity of SA-β-gal. (D) Quantification of activities of SA-β-gal in shCON or shSPHK1-expressing hAD-SCs. ***P<0.001. (E) Depletion of SPHK1 causes increase in the levels of senescence markers. β-Actin serves as a loading control. (F) Elevated levels of key sphingolipid species induced by knockdown of SPHK1. *P<0.05 and **P<0.01, compared to control shRNA-expressing cells.

Fig.4. SPHK1 knockdown-accelerated cellular senescence is attenuated by inhibition of ceramide synthesis and concurrent supplementation of S1P. (A) No rescue of reduced proliferation capacity of SPHK1-depleted hAD-SCs by co-treatment with S1P and FB_1. **P<0.01. (B) Co-treatment with S1P and FB_1 lowers the activity of SA-β-gal enhanced by depletion of SPHK1. (C) Quantification of activities of SA-β-gal in shCON or shSPHK1-expressing hAD-SCs which were co-treated with S1P and FB_1 or not. ***P<0.001.

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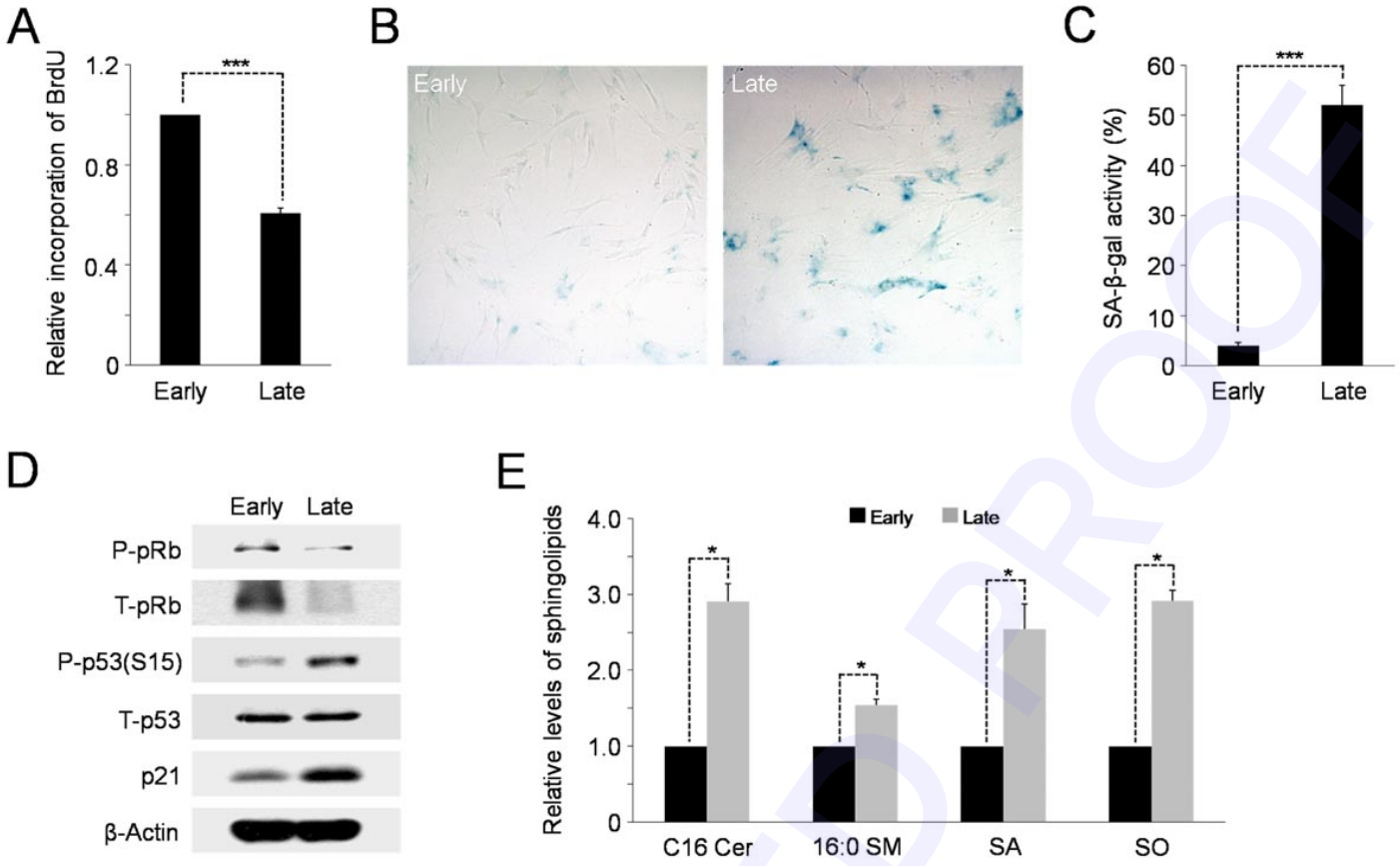
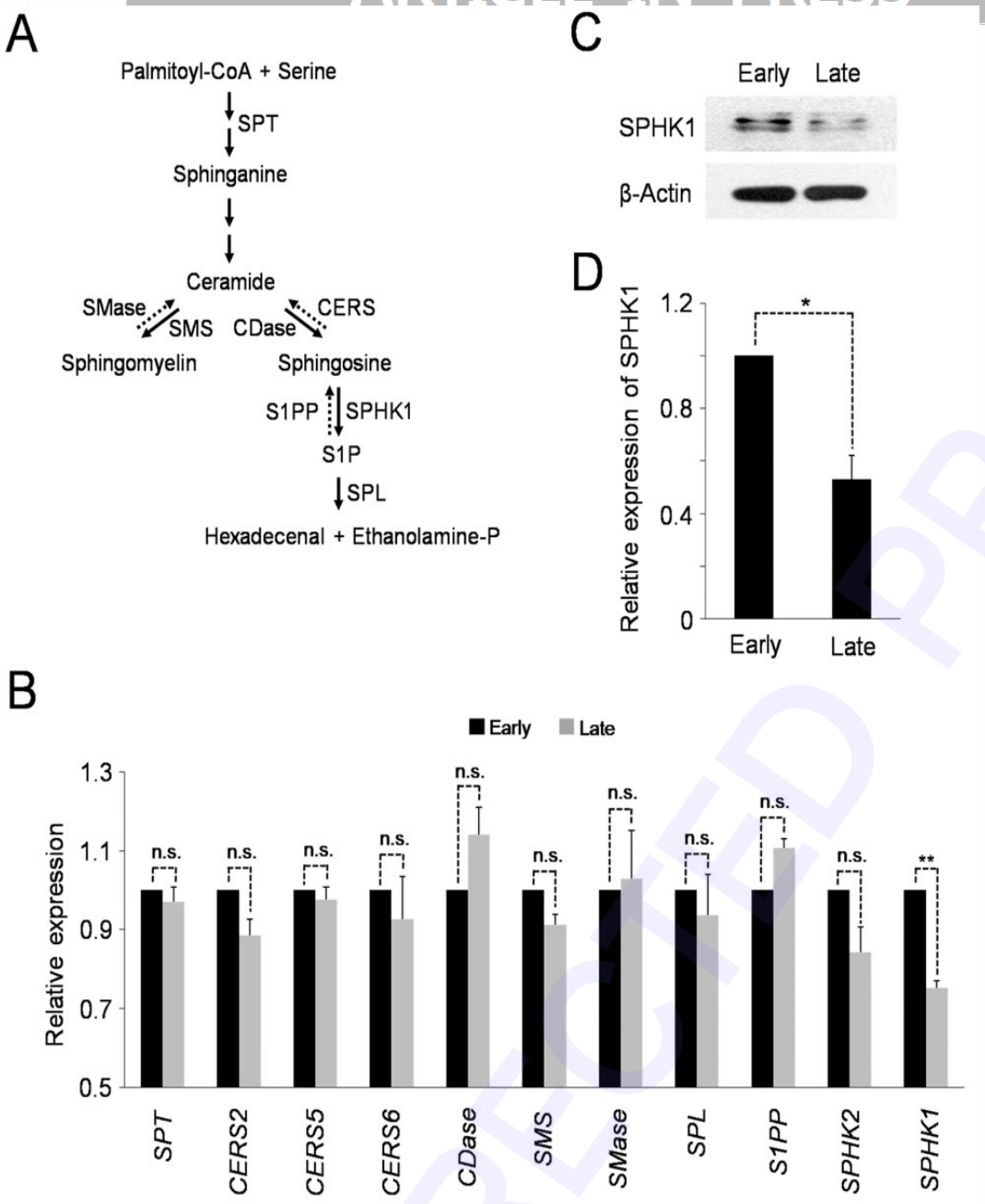


Fig. 1. Figure 1



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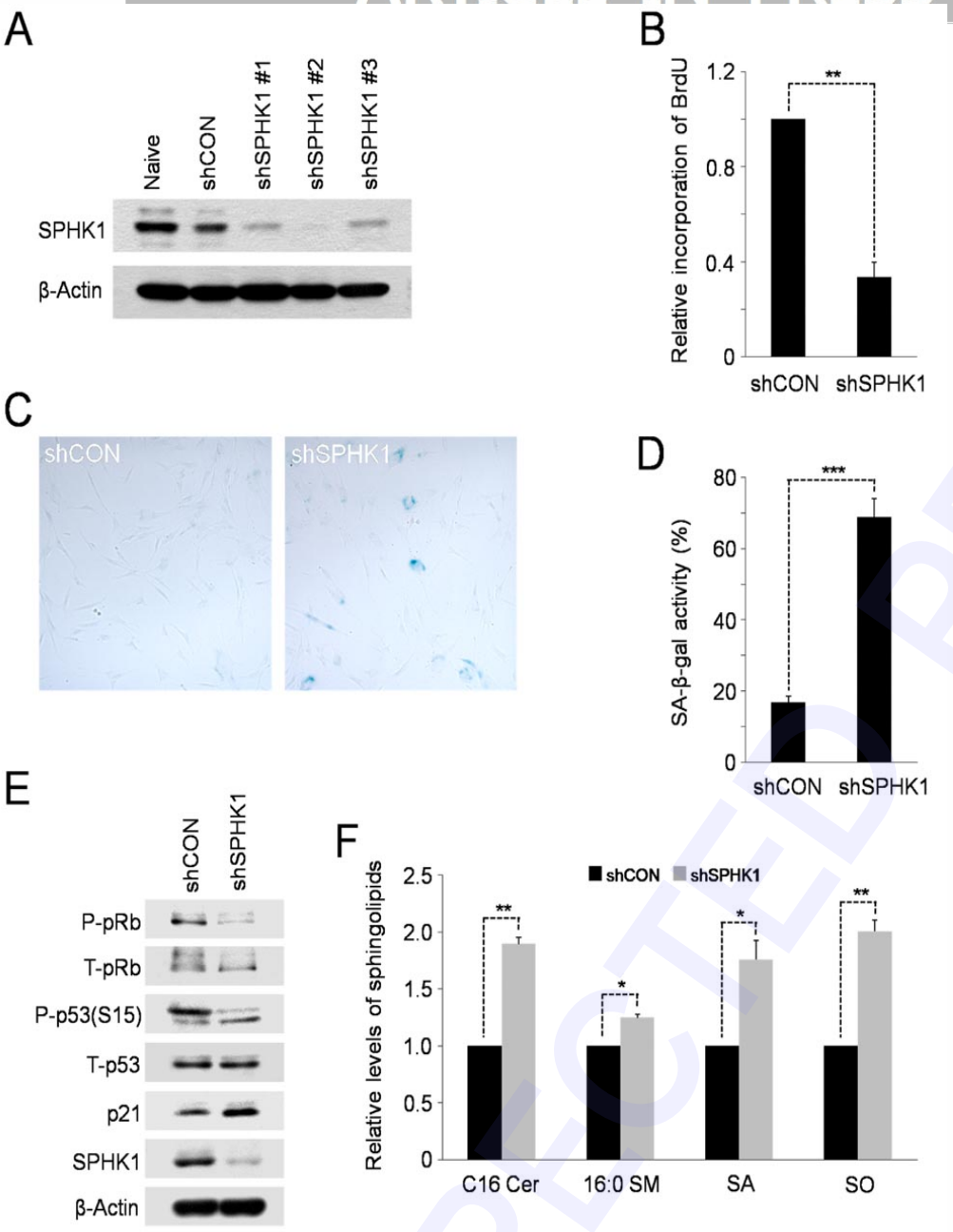


Fig. 3. Figure 3

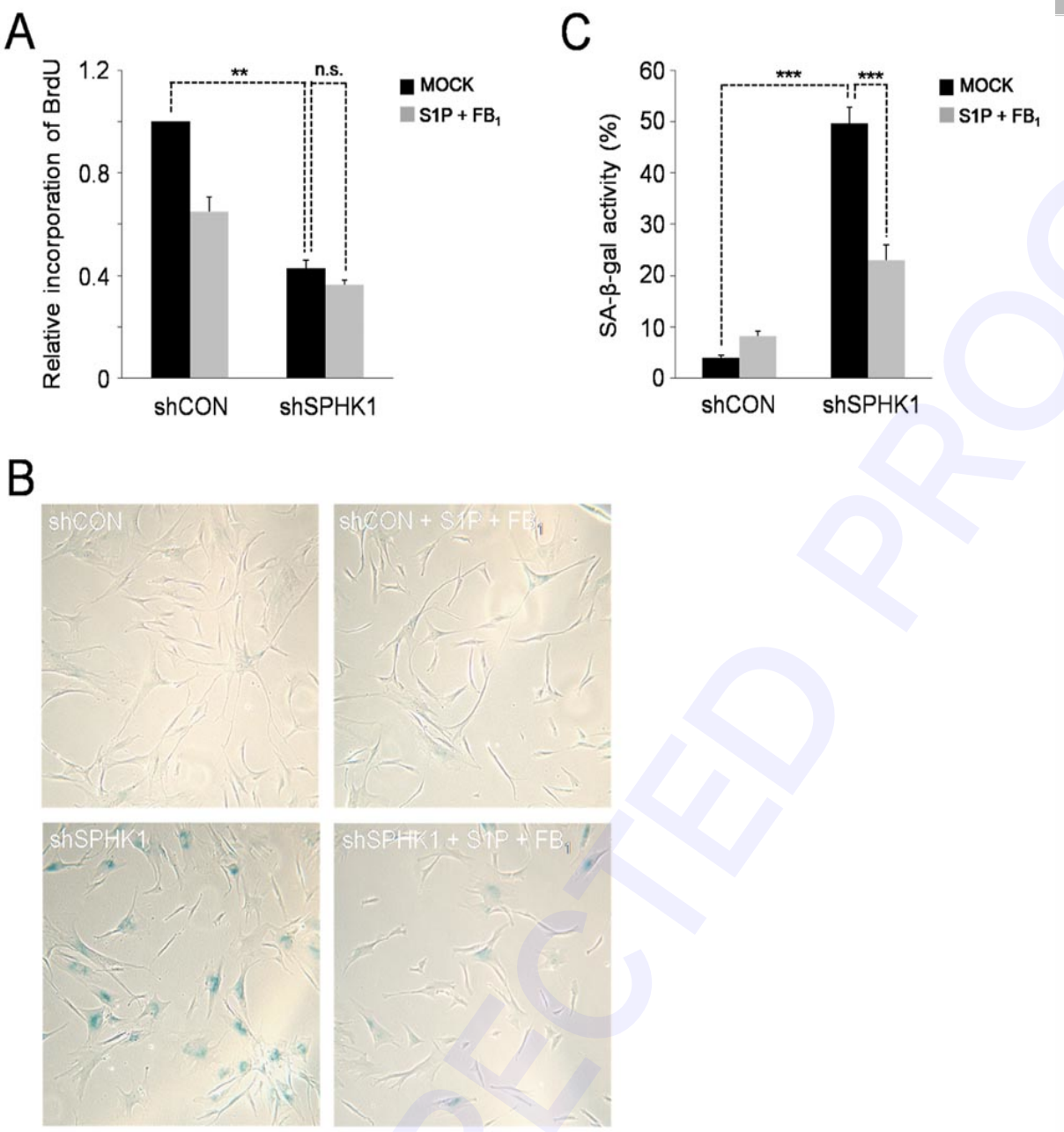


Fig. 4. Figure 4

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

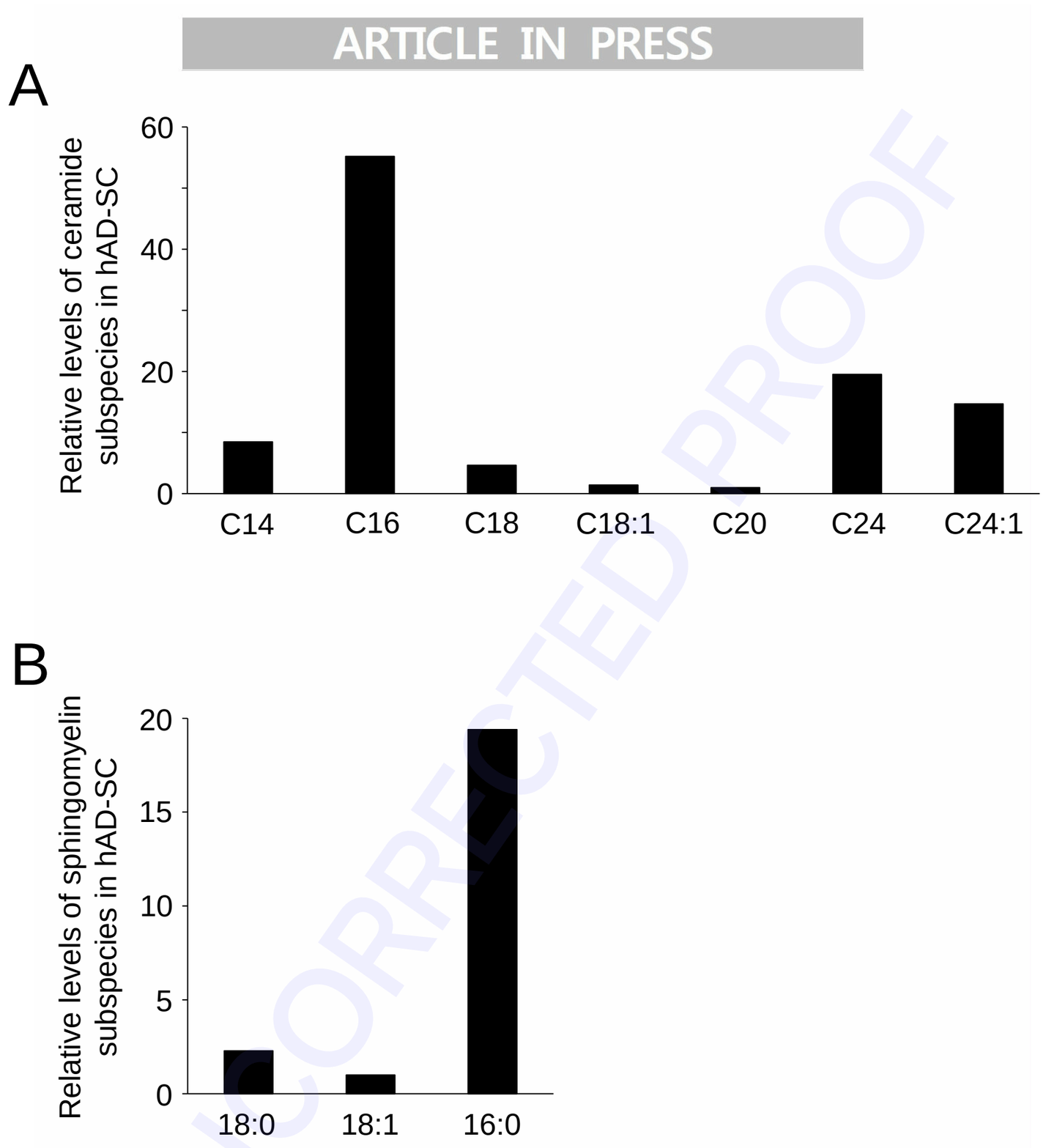
For LC-MS/MS, hAD-SCs were seeded in T175 flasks at a cell density of 4 x 10^5 per flask and incubated for 4 days when the cell density reaches 2.5 x 10^6 per flask. Cells were dissociated using trypsin-EDTA and subsequently subjected to centrifugation, followed by resuspension of the pelleted cells 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

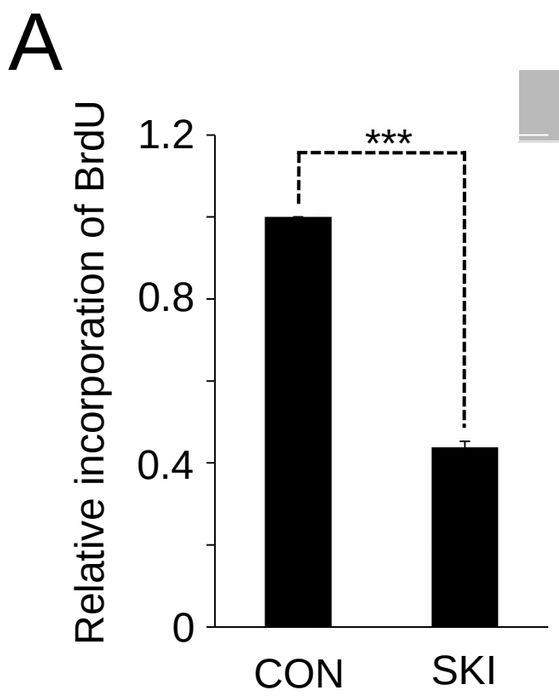
min), 30 % of A (t = 15 min), 10 % of A (t = 22 min), 10 % of A (t = 25 min), 50 % of A (t = 25.1 min), 50 % of A (t = 30 min) with 200 µl/min at 35 °C. The MRM (multiple reaction monitoring) mode was used in the positive ion mode and the peak area of the extracted ion chromatogram (EIC) corresponding 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 ($r^2 \geq 0.99$).

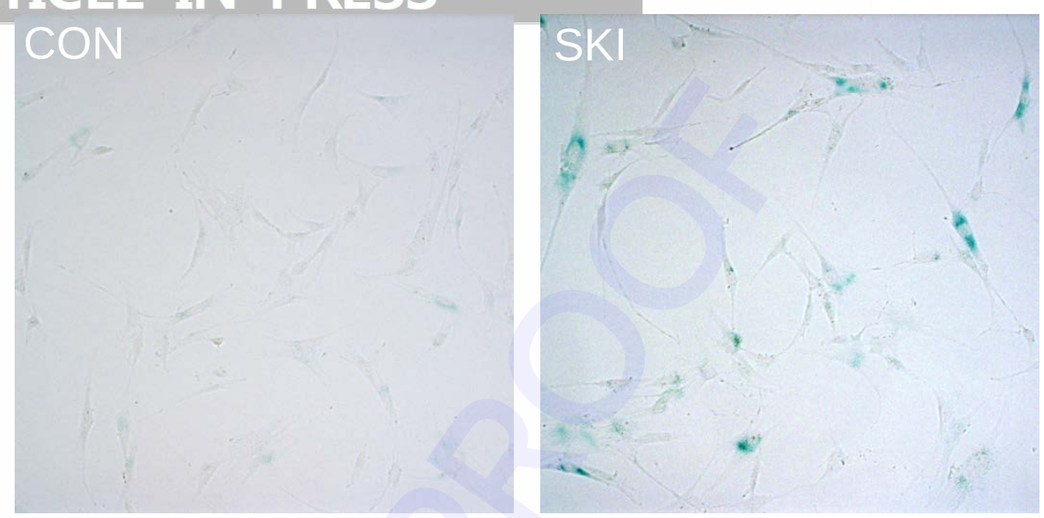
Statistical analysis

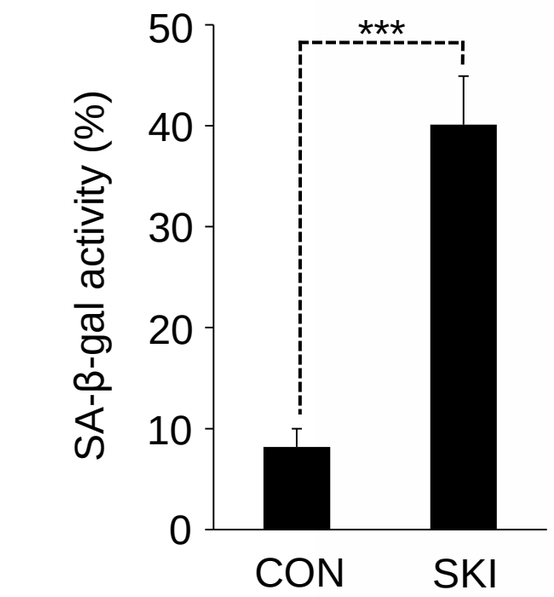
All experimental data were analyzed using the GraphPad Prism 5.01 software program (San Diego, CA, USA) and are expressed as the mean ± standard error (SE) from three independent experiments. $P < 0.05$ was considered statistically significant.

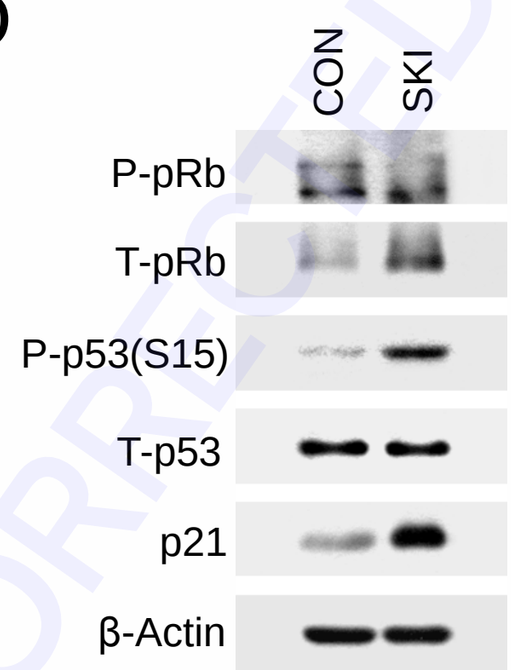


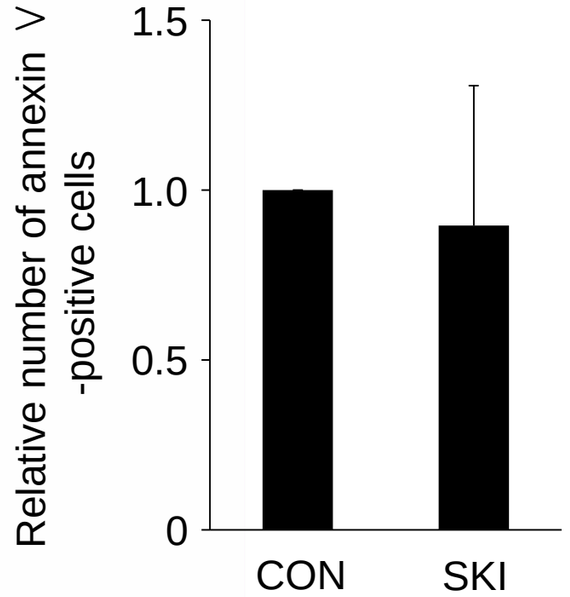
Supplementary Fig. 1. (A, B) LC-MS/MS analysis showing the relative amounts of ceramide (A) and sphingomyelin (B) subspecies in hAD-SCs.



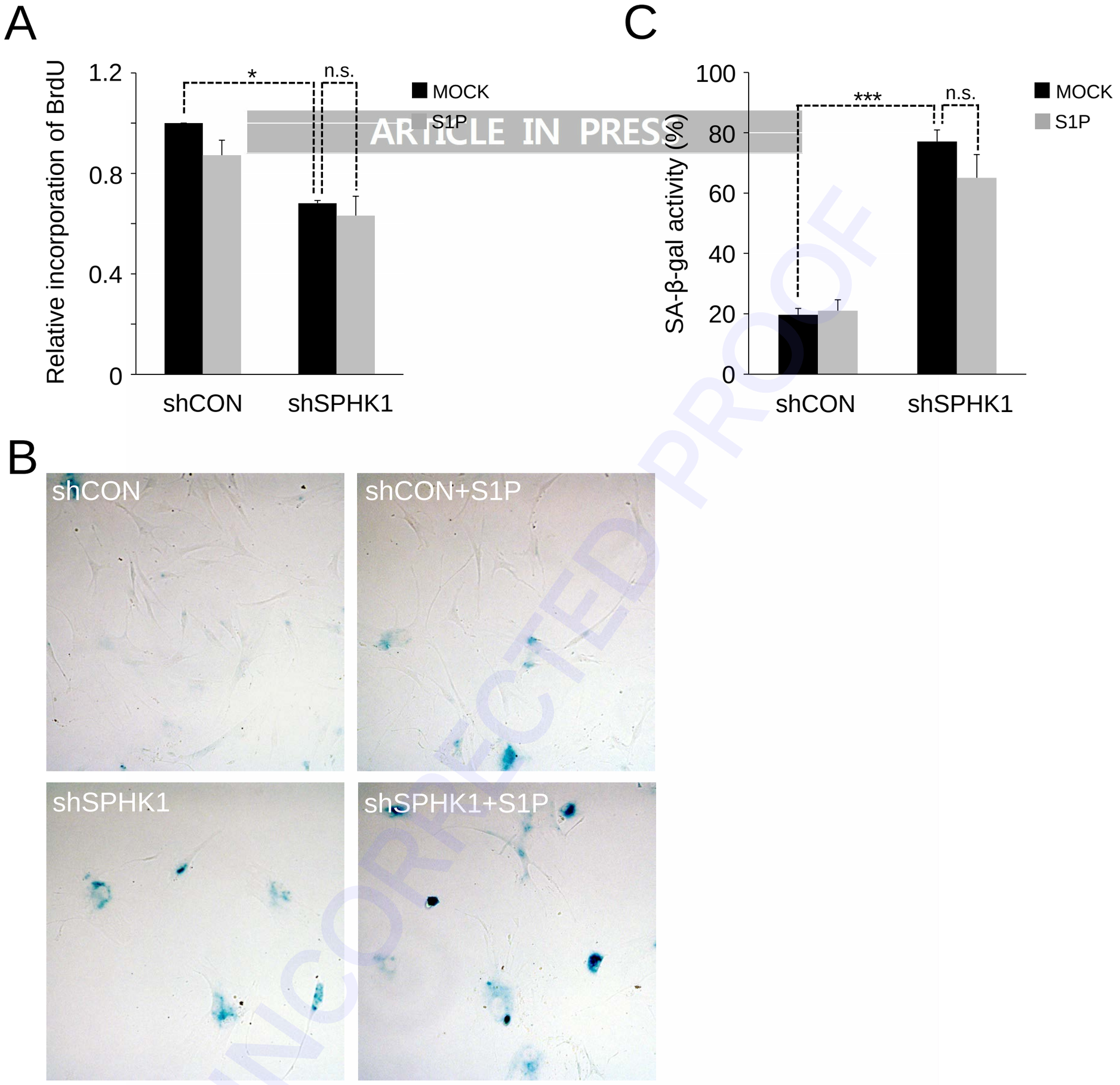




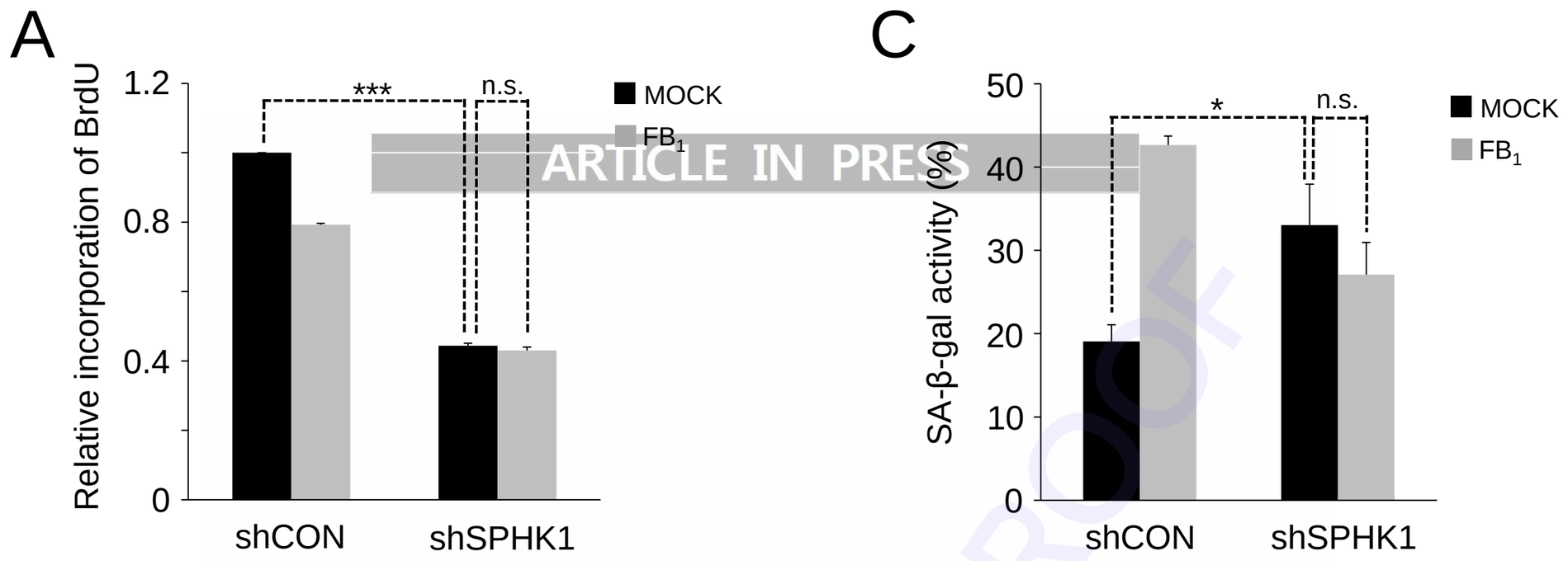


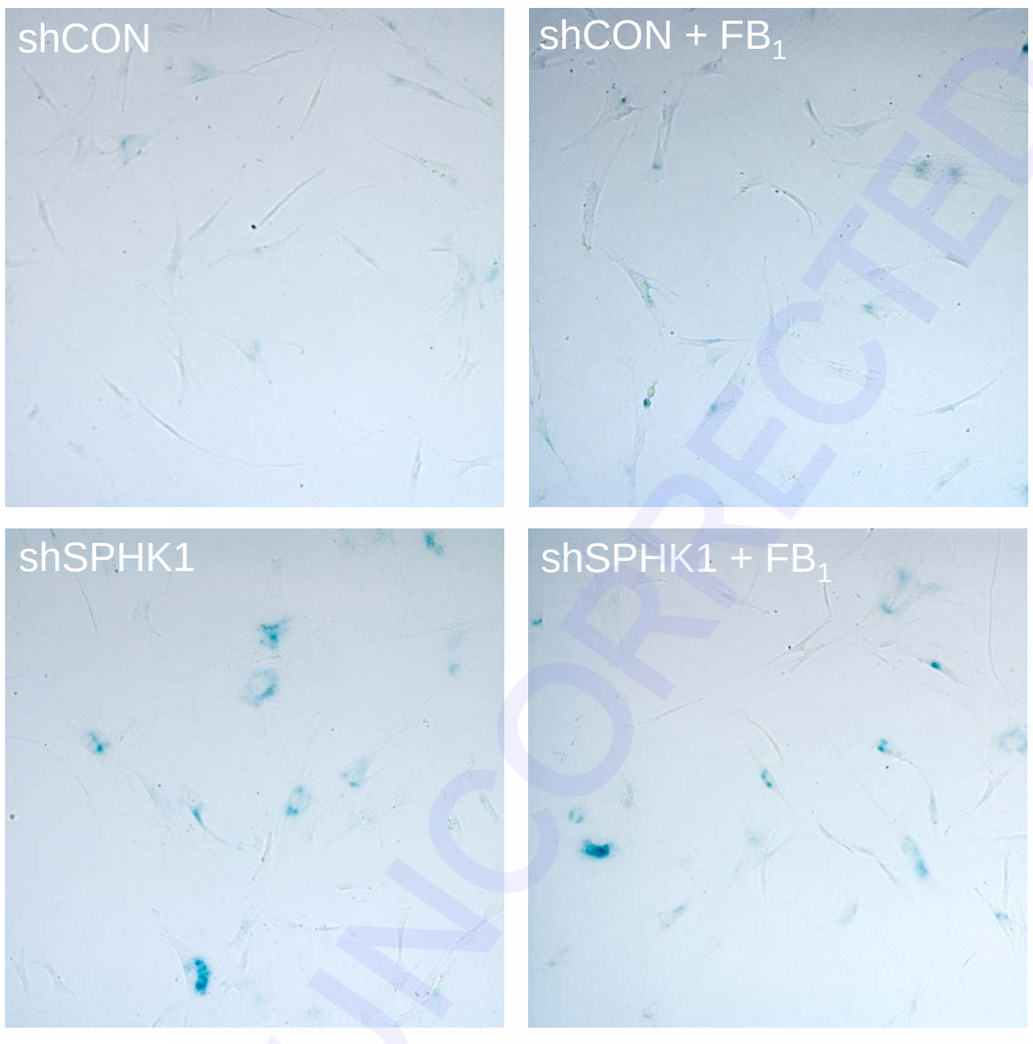


Supplementary Fig. 2. Inhibition of enzymatic activity of SPHK1 accelerates cellular senescence. (A) Treatment with SKI interferes with proliferation of hAD-SCs. ***P<0.001. (B) Activity of SA-β-gal is augmented by treatment with SKI. (C) Quantification of activities of SA-β-gal in DMSO-treated control (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