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Interference with sphingosine kinase-1 reduces the hydrogen peroxide-induced oxidative stress damage in melanocytes through four and a half LIM domains 2

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Abstract. Vitiligo is featured by manifestation of white maculae and primarily results from oxidative stress. Sphingosine kinase-1 (SPHK1) participates in oxidative stress. This paper was devised to explore the role of SPHK1 in vitiligo and to disclose the mechanism. PIG1 cell viability was appraised utilizing cell counting kit-8 assay while Western blot detected SPHK1 and four and a half LIM domains 2 (FHL2). The transduction efficacy of small interfering RNA (siRNA)-SPHK1, siRNA-FHL2 and pcDNA3.1 plasmid overexpressing FHL2 (Ov-FHL2) was checked using Western blot. Flow cytometry detected cell apoptotisis. Western blot detected mitochondrial cytochrome c (Mit-Cyt-c) and cytosolic cytochrome c (Cyto-Cyt-c). Dichloro-dihydro-fluorescein diacetate (DCFH-DA) detected reactive oxygen species (ROS) activity while oxidative stress markers were evaluated using corresponding assay kits. SPHK1 expression was discovered to be increased in hydrogen peroxide (H_2O_2)-challenged PIG1 cells and SPHK1 interference alleviated H_2O_2-challenged viability damage, apoptosis, oxidative stress and FHL2 expression in PIG1 cells. FHL2 depletion could suppress viability damage, apoptosis and oxidative stress in H_2O_2-challenged PIG1 cells. Rescue experiments demonstrated that the suppressive impacts of SPHK1 deficiency on PIG1 cell viability, apoptosis and oxidative stress induced by H_2O_2 were offset by FHL2 overexpression. Collectively, SPHK1 knockdown protected against vitiligo *via* the regulation of FHL2.

Key words: SPHK1 — Melanocytes — Oxidative stress — FHL2 — Vitiligo

Abbreviations: CAT, catalase; CCK-8, cell counting kit-8; Cyto-Cyt-c, cytosolic cytochrome c; DCFH-DA, dichloro-dihydro-fluorescein diacetate; FBS, fetal bovine serum; FHL2, four and a half LIM domains 2; MDA, malondialdehyde, SOD, superoxide dismutase; Mit-Cyt-c, mitochondrial cytochrome c; Ov-FHL2, pcDNA3.1 plasmid overexpressing FHL2; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; siRNA, small interfering RNA; SPHK1, sphingosine kinase-1.

Introduction

As an obtained chronic depigmenting disorder of the skin, vitiligo manifests white patches resulting from the loss of functional melanocytes and brings huge negative influences to patients suffering from vitiligo (Bergqvist and Ezzedine 2021). Previous study has proposed various mechanisms for vitiligo, such as oxidative stress, melanocyte-detachment, autoimmune and inflammatory mediator (Bergqvist and Ezzedine 2020). Oxidative stress is believed to be primary responsible for vitiligo among above-mentioned possible mechanisms (An et al. 2021). Besides, it has been revealed that reactive oxygen spe-

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cies (ROS) activity was discovered to be ascended in the epidermis of vitiligo patients (Delmas and Larue 2019). Furthermore, accumulated hydrogen peroxide (H_2O_2) production can be found in vitiligo patients at advanced stage (Schallreuter et al. 1999).

Sphingosine kinase-1 (SPHK1), which belongs to the SPHK family, can catalyze the generation of sphingosine-1-phosphate (S1P) (Yao et al. 2020). SPHK1 has been extensively implicated in oxidative stress. In myocardial fibrosis, the interference of SPHK1 can improve the viability and suppress oxidative stress in oxygen and glucose deprivation (OGD)-challenged treated HL-1 cells (Chen et al. 2023). In addition, Qiang et al. (2019) have clarified that SPHK1 depletion can mitigate apoptosis and oxidative stress in mice with hepatic ischemia/reperfusion. According to data set GSE53148, SPHK1 expression is elevated in the skin tissues of vitiligo patients (FC = 1.7, $p < 0.05$). However, it remains elusive how SPHK1 functions in vitiligo.

Four and a half LIM domains 2 (FHL2) is a critical component of LIM-only protein family and guarantees the assembly of multi-protein complexes for a diverse range of cell activities, such as cell viability, apoptosis, along with proliferation (Tran et al. 2016). A growing body of studies have illustrated that FHL2 plays a vital regulatory role in various human diseases, including cardiovascular disease as well as different kind of cancers (van de Pol et al. 2020; Jiao et al. 2022). Of note, Hu et al. (2022) have illuminated that FHL2 might be a potent therapeutic target for vitiligo. Additionally, Hitpredict database (https://www.hitpredict.org/) predicted that SPHK1 can bind to FHL2 and the binding relationship is confirmed by HDOCK Server (http://hdock.phys.hust.edu.cn/). However, whether SPHK1 functions in vitiligo *via* FHL2 is obscure.

In summary, this study was devoted to investigating the protective mechanisms of SPHK1 in H_2O_2-stimulated oxidative stress damage in melanocytes through FHL2.

Materials and Methods

Cell culture and treatment

Medium 254, which was decorated with Human Melanocyte Growth Supplement and 5% fetal bovine serum (FBS) was used for the maintaining of PIG1 normal human skin melanocytes (iCell Bioscience Inc, Shanghai). The conditions set for cultivation were 37°C and 5% CO_2. For simulating oxidative stress damage *in vitro*, PIG1 cells were challenged by H_2O_2 with varying concentrations (0.5, 0.8 and 1 mM) for 24 h (An et al. 2021). PIG1 cells were pre-treated with SPHK1 inhibitor PF543 at different concentrations (1 and 2 μM) for 24 h (Cheresh et al. 2020).

Cell transfection

The construction of small interfering RNAs (siRNAs) specific to SPHK1 (siRNA-SPHK1-1/2) and FHL2 (siRNA-FHL2-1/2), the corresponding negative control (siRNA-NC), pcDNA3.1 plasmid overexpressing FHL2 (Ov-FHL2) and the empty vector (Ov-NC) were done by Shanghai GenePharma Co., Ltd. The introduction of these recombinants into PIG1 cells was accomplished by virtue of Lipofectamine 2000 transfection reagent (Invitrogen) in light of the operating manual. Following 48 h, PIG1 cells were collected for follow-up studies. The target sequences of SPHK1-1: 5'-GCAGGCAUAUGGAGUAUGATTUCAUACUCCAUAUGCCUGCTT-3'; SPHK1-2: 5'-GCGUCAUGCAUCUGUUCUATTUAGAACAGAUGCAUGACGCTT-3'; siRNA-FHL2-1: 5'-ATCTCTCTTTGGCAAGAAGTACA-3'; siRNA-FHL2-2: 5'-GTGACTTGTATGCCAAGAAGTGT-3'; siRNA-NC: 5'-AAGACAUUGUGUGUCCGCCTT-3'.

Cell counting kit-8 (CCK-8) assay

To appraise the impacts of H_2O_2 challenge on PIG1 cell viability, CCK-8 assay was initially implemented. In short, the PIG1 cells that inoculated into 96-well plates underwent 24-h cultivation. Afterwards, *per* well was decorated with 10 μl of CCK-8 reagent to cultivate cells for extra 3 h with 5% CO_2 at 37°C (Pan et al. 2023). The optical density value was appraised utilizing a microplate reader at 450 nm.

Western blot

The concentration of proteins that isolated from sample PIG1 cells utilizing radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio) was quantified employing bicinchoninic acid (BCA) protein assay kits in accordance with the recommended specifications. The proteins, which were subjected to 8% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation, were loaded to polyvinylidene fluoride (PVDF) membranes. Prior to the successive exposure to primary antibodies specific to SPHK1 (cat. no. ab302714; 1:1000, Abcam), cytochrome c (Cyt-c; cat. no. ab76107; 1:500, Abcam), FHL2 (cat. no. ab202584; 1:1000, Abcam) or GAPDH (cat. no. ab9485; 1:2500, Abcam) at 4°C overnight and horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (cat. no. ab6721; 1:2000, Abcam), the membranes were sealed by 5% bovine serum albumin (BSA). The protein blots got visualized with the adoption of electrochemiluminescence (ECL) Detection Reagent (Yeasen Biotech) and grayscale values was quantified employing Image J software (Version 1.49; National Institutes of Health).

Flow cytometry

Following the rinse with pre-cold phosphate buffered saline (PBS), PIG1 cells underwent the resuspension in 100 µl binding buffer and were introduced to 2 µl Annexin V-fluorescein isothiocyanate (Annexin V-FITC; 20 µg/ml, Sigma) on ice away from light. Thereafter, PIG1 cells were subjected to propidium iodide (PI, BD Biosciences) staining. The appraisement of PIG1 cell apoptosis was carried out utilizing a fluorescence activated cells sorting (FACS) Calibur Flow cytometer (BD Bioscience) in light of standard protocol.

Measurement of MDA, SOD and CAT

The activities of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) in cell supernatants were estimated by means of MDA assay kits (cat. no. S0131S), SOD assay kits (cat. no. S0109) and CAT (cat. no. S0082), all of which were provided by Beyotime Institute of Biotechnology (Shanghai, China), in accordance with the suggested specifications.

Measurement of ROS

ROS assay kits (cat. no. S0033S) that procured from Beyotime Institute of Biotechnology were adopted for the estimation of ROS activity. In short, PIG1 cells were introduced to dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe for 20 min of staining. Subsequently, the fluorescence intensity of PBS-rinsed PIG1 cells was estimated employing a fluorescence microscope with excitation and emission at 488 nm and 522 nm, respectively.

Co-immunoprecipitation (Co-IP)

The proteins that isolated from sample PIG1 cells utilizing IP lysis buffer underwent the cultivation with antibodies specific to SPHK1 (cat. no. ab302714; 1:30; Abcam), FHL2 (cat. no. ab202584; 1:30; Abcam), rabbit-IgG (cat. no. ab313801; 1:30; Abcam) and Protein A/G PLUS-Agarose overnight at 4°C. Then, the PBS-rinsed precipitated proteins underwent the suspension, boiling for 5 min and elusion from beads. At last, the collection of eluates was implemented through magnetic separation and Western blot was adopted for analysis.

Statistical analysis

The collected experimental data that got analyzed with GraphPad Prism 8.0 (GraphPad Software, Inc.) were exhibited in the way of mean ± standard deviation. For the exhibition of comparisons between two groups, Student's *t*-test was employed while one-way analysis of variance, together with Bonferroni *post hoc* test was adopted for the demonstration of differences among three or more groups. $p < 0.05$ was an indicator of statistical significance.

Results

SPHK1 expression was upregulated in H_2O_2-challenged PIG1 cells

According to data set GSE53148, SPHK1 expression was markedly elevated in the skin tissues of vitiligo patients (Fig. 1A). In order to figure the effects of H_2O_2 with varying concentrations (0.5, 0.8 and 1 mM) on PIG1 cell viability, CCK-8 assay was initially carried out. It was noted that PIG1 cell viability was conspicuously reduced by H_2O_2 challenge in a concentration-dependent manner (Fig. 1B). Western blot was employed for the estimation of SPHK1 expression in H_2O_2-challenged PIG1 cells and it was discovered that H_2O_2 stimulation dose-dependently elevated the protein content of SPHK1 in PIG1 cells (Fig. 1C). Considering the prominent suppressive effects of H_2O_2 at a concentration of 1 mM on PIG1 cell viability, 1 mM H_2O_2 was selected for follow-up studies.

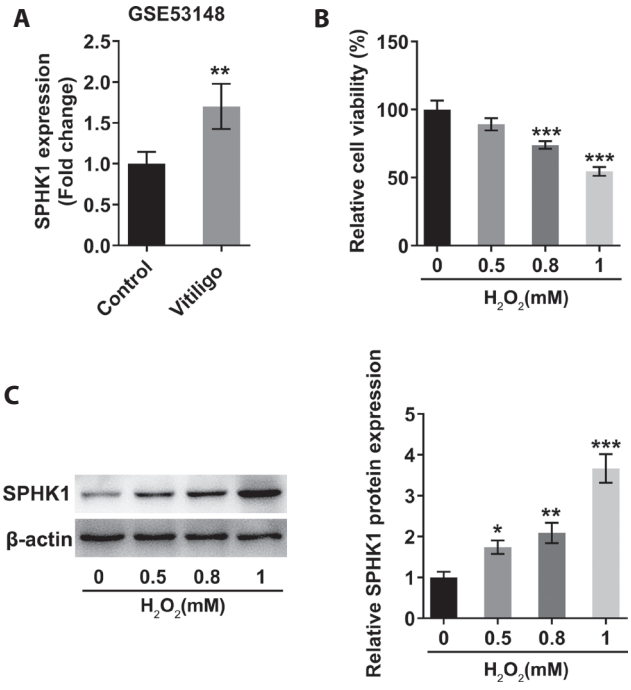
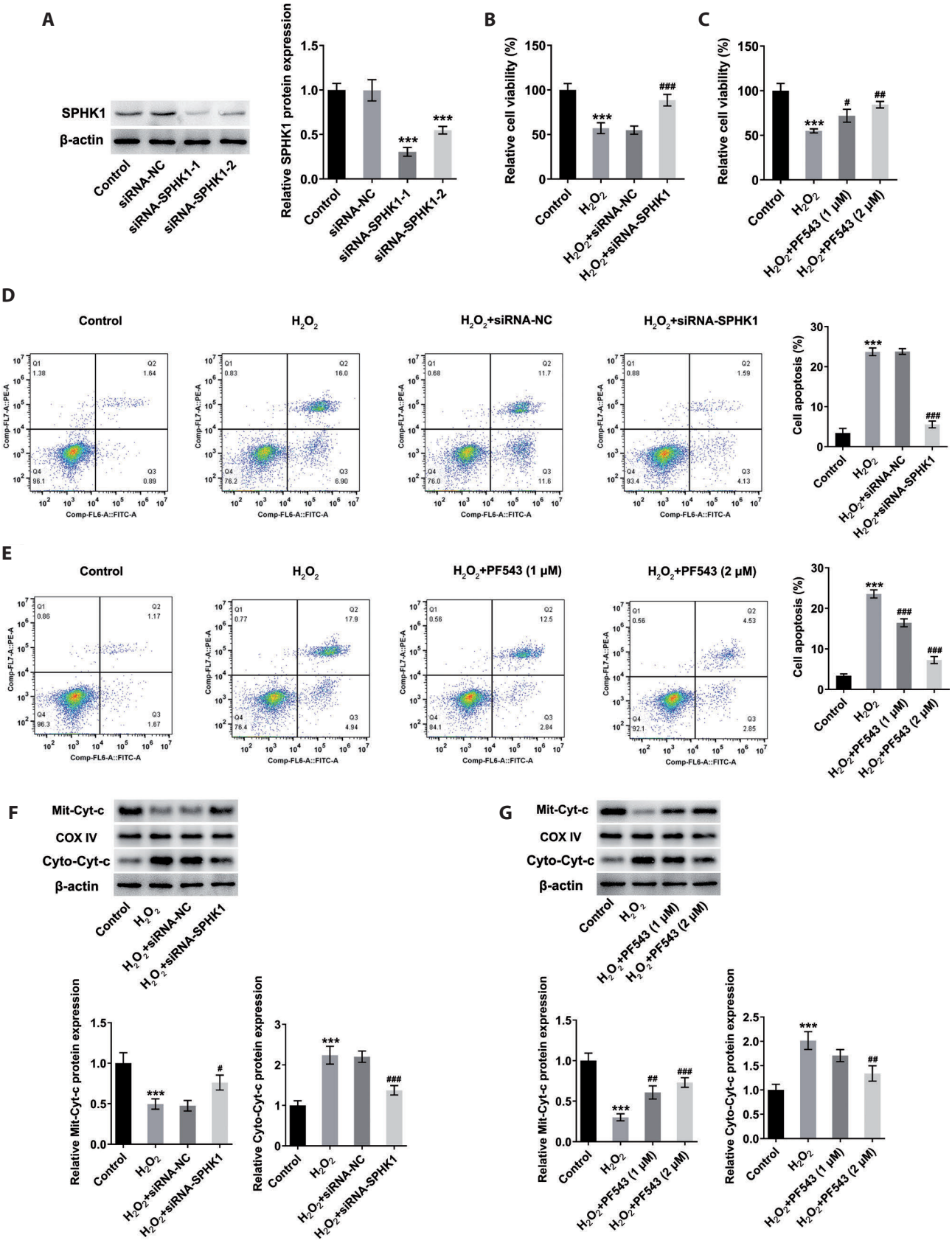


Figure 1. SPHK1 expression was upregulated in H_2O_2-challenged PIG1 cells. **A.** According to data set GSE53148, SPHK1 expression was upregulated in the skin tissues of vitiligo patients. ** $p < 0.01$ *vs.* Control. **B.** The viability of H_2O_2-challenged PIG1 cells was appraised employing CCK-8 assay. **C.** SPHK1 expression in H_2O_2-challenged PIG1 cells was appraised employing Western blot. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* H_2O_2 (0 mM).


A
SPHK1
β-actin
Control
siRNA-NC
siRNA-SPHK1-1
siRNA-SPHK1-2
Relative SPHK1 protein expression
B
Relative cell viability (%)
Control
H2O2
H2O2+siRNA-NC
H2O2+siRNA-SPHK1
C
Relative cell viability (%)
Control
H2O2
H2O2+PF543 (1 μM)
H2O2+PF543 (2 μM)
D
Control
H2O2
H2O2+siRNA-NC
H2O2+siRNA-SPHK1
Comp-FL7-A::PE-A
Comp-FL6-A::FITC-A
Cell apoptosis (%)
E
Control
H2O2
H2O2+PF543 (1 μM)
H2O2+PF543 (2 μM)
Cell apoptosis (%)
F
Mit-Cyt-c
COX IV
Cyto-Cyt-c
β-actin
Relative Mit-Cyt-c protein expression
Relative Cyto-Cyt-c protein expression
G
Mit-Cyt-c
COX IV
Cyto-Cyt-c
β-actin
Relative Mit-Cyt-c protein expression
Relative Cyto-Cyt-c protein expression

SPHK1 interference alleviated the viability damage and apoptosis in H_2O_2-challenged PIG1 cells

With the aim of declining SPHK1 expression, siRNA-SPHK1 was introduced to PIG1 cells and the transduction efficacy was examined utilizing Western blot. In comparison with the siRNA-NC group, SPHK1 expression in PIG1 cells was distinctly eliminated by siRNA-SPHK1 (Fig. 2A). It was worthwhile to mention that SPHK1 had lower expression in siRNA-SPHK1-1 group; therefore, siRNA-SPHK1-1 (hereinafter referred as siRNA-SPHK1) was selected for follow-up studies. PIG1 cells were divided into Control, H_2O_2, H_2O_2+siRNA-NC and H_2O_2+siRNA-SPHK1 groups. By contrast with the Control group, H_2O_2 stimulation remarkably diminished the viability of PIG1 cells, which was then partially revived by SPHK1 interference (Fig. 2B). To better elucidate the role of SPHK1 in PIG1 cells, we also adopted SPHK1 inhibitor PF543 to treat PIG1 cells and cells were divided into Control, H_2O_2, H_2O_2+PF543 (1 μM) and H_2O_2+PF543 (2 μM) groups. Similarly, SPHK1 inhibitor PF543 improved the viability of H_2O_2-challenged PIG1 cells (Fig. 2C). As we all know, the apoptosis of melanocytes can contribute to vitiligo. The role of SPHK1 in PIG1 cell apoptosis was explored through the implementation of flow cytometry and it was discovered that the facilitated apoptosis in PIG1 cells due to H_2O_2 stimulation was evidently suppressed by SPHK1 depletion or SPHK1 inhibitor PF543 (Fig. 2D,E). Cyt-c is a critical initiator the caspases triggering cell apoptosis. The effects of SPHK1 depletion on mitochondrial cytochrome c (Mit-Cyt-c) and cytosolic cytochrome c (Cyto-Cyt-c) were explored hereby. Relative to the Control group, H_2O_2 induction descended Mit-Cyt-c expression whereas ascended Cyto-Cyt-c expression, which were all reversed by SPHK1 deficiency or SPHK1 inhibitor PF543 (Fig. 2F,G).

SPHK1 interference alleviated oxidative stress in H_2O_2-challenged PIG1 cells

Excessive ROS production can be discovered in melanocytes of vitiligo patients. In the beginning, ROS activity was assessed utilizing DCFH-DA. It was noted that the elevated ROS activity in H_2O_2 group was diminished by SPHK1 depletion or SPHK1 inhibitor PF543 (Fig. 3A,E). Oxidative stress has been supposed to be an underlying contributor to in the pathogenesis of vitiligo. Therefore, we also appraised the activities of oxidative stress markers in this section. H_2O_2 challenge ascended MDA activity whereas descended the activities of SOD and CAT in comparison with the Control group, which were all reversed by SPHK1 interference or SPHK1 inhibitor PF543 (Fig. 3B–D and 3F–H).

SPHK1 interference inhibited FHL2 expression in H_2O_2-challenged PIG1 cells

According to HDOCK Server, SPHK1 was confirmed to bind to FHL2 (Fig. 4A). To figure out the regulatory role of SPHK1 in FHL2, the protein content of FHL2 in H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 or treated with PF543 was investigated. As Figure 4B and C depicted, SPHK1 knockdown or SPHK1 inhibitor PF543 evidently reduced the increased FHL2 expression in PIG1 cells with H_2O_2 induction. The interaction of SPHK1 with FHL2 was attested utilizing Co-IP assay and it was demonstrated that SPHK1 could bind to FHL2 in H_2O_2-insulted PIG1 cells (Fig. 4D).

FHL2 depletion suppressed the viability damage, apoptosis and oxidative stress in H_2O_2-challenged PIG1 cells

With the purpose of diminishing FHL2 expression, siRNA-FHL2 was introduced to PIG1 cells and Western blot was adopted for the examination of transduction efficacy. Relative to the siRNA-NC group, FHL2 expression was dramatically reduced following the transfection with siRNA-FHL2 (Fig. 5A). Of note, FHL2 had lower expression in siRNA-FHL2-1 group, in this way, siRNA-FHL2-1 (hereinafter referred as siRNA-FHL2) was selected for follow-up studies. The impacts of siRNA-FHL2 on PIG1 cell viability was investigated through the implementation of CCK-8 assay. As Figure 5B displayed, the declined PIG1 cell viability because of H_2O_2 challenge was partially revived following the introduction of siRNA-FHL2. Besides, the facilitated cell apoptosis in H_2O_2 group was suppressed in H_2O_2+siRNA-FHL2 group (Fig. 5C). Moreover, H_2O_2 challenge elevated ROS and MDA activities whereas diminished SOD and

◀ **Figure 2.** SPHK1 interference alleviated the viability damage and apoptosis in H_2O_2-challenged PIG1 cells. **A.** The transfection efficacy of siRNA-SPHK1 was estimated employing Western blot. *** $p < 0.001$ *vs.* siRNA-negative control (NC). **B.** The viability of H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 was appraised employing CCK-8 assay. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2+siRNA-NC. **C.** The viability of H_2O_2-challenged PIG1 cells with PF543 treatment was appraised employing CCK-8 assay. *** $p < 0.001$ *vs.* Control, # $p < 0.05$ and ## $p < 0.01$ *vs.* H_2O_2. **D.** The apoptosis of H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 was appraised employing flow cytometry. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2+siRNA-NC. **E.** The apoptosis of H_2O_2-challenged PIG1 cells with PF543 treatment was appraised employing flow cytometry. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2. **F.** The protein contents of Mit-Cyt-c and Cyto-Cyt-c in H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 were appraised employing Western blot. *** $p < 0.001$ *vs.* Control, # $p < 0.05$ and ### $p < 0.001$ *vs.* H_2O_2+siRNA-NC. **G.** The protein contents of Mit-Cyt-c and Cyto-Cyt-c in H_2O_2-challenged PIG1 cells with PF543 treatment were appraised employing Western blot. *** $p < 0.001$ *vs.* Control, ## $p < 0.01$ and ### $p < 0.001$ *vs.* H_2O_2.

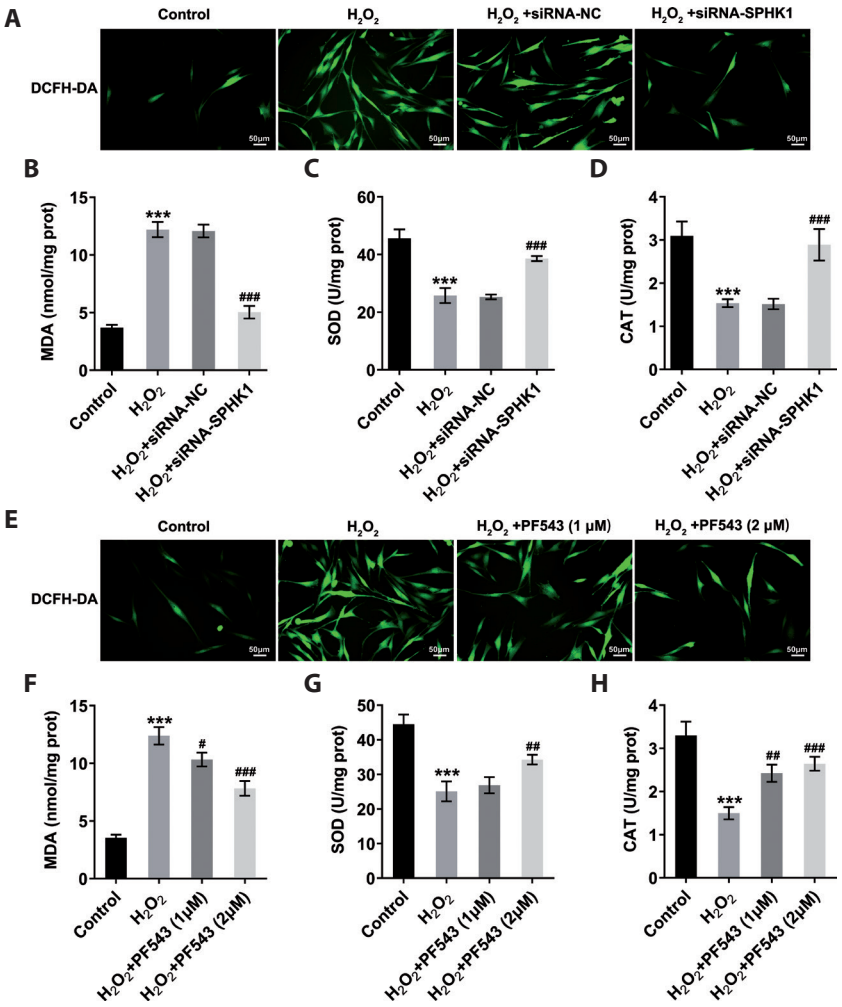


Figure 3. SPHK1 interference alleviated oxidative stress in H_2O_2-challenged PIG1 cells. **A.** ROS activity in H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 was appraised employing DCFH-DA staining. The activities of MDA (**B**), SOD (**C**) and CAT (**D**) in H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 were appraised employing corresponding assay kits. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2+siRNA-NC. **E.** ROS activity in H_2O_2-challenged PIG1 cells with PF543 treatment was appraised employing DCFH-DA staining. The activities of MDA (**F**), SOD (**G**) and CAT (**H**) in H_2O_2-challenged PIG1 cells with PF543 treatment were appraised employing corresponding assay kits. *** $p < 0.001$ *vs.* Control, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ *vs.* H_2O_2.

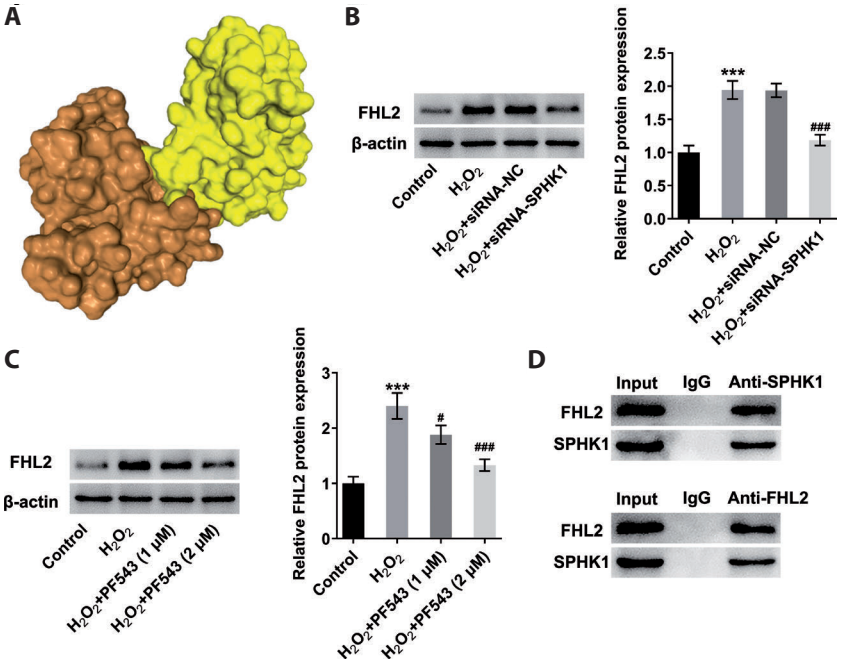


Figure 4. SPHK1 interference inhibited FHL2 expression in H_2O_2-challenged PIG1 cells. **A.** According to HDOCK Server, SPHK1 can bind to FHL2. **B.** FHL2 expression in H_2O_2-challenged PIG1 cells transfected with siRNA-SPHK1 was appraised employing Western blot. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2+siRNA-NC. **C.** FHL2 expression in H_2O_2-challenged PIG1 cells with PF543 treatment was appraised employing Western blot. *** $p < 0.001$ *vs.* Control, # $p < 0.05$ and ### $p < 0.001$ *vs.* H_2O_2. **D.** The binding of SPHK1 with FHL2 was confirmed utilizing Co-IP assay.

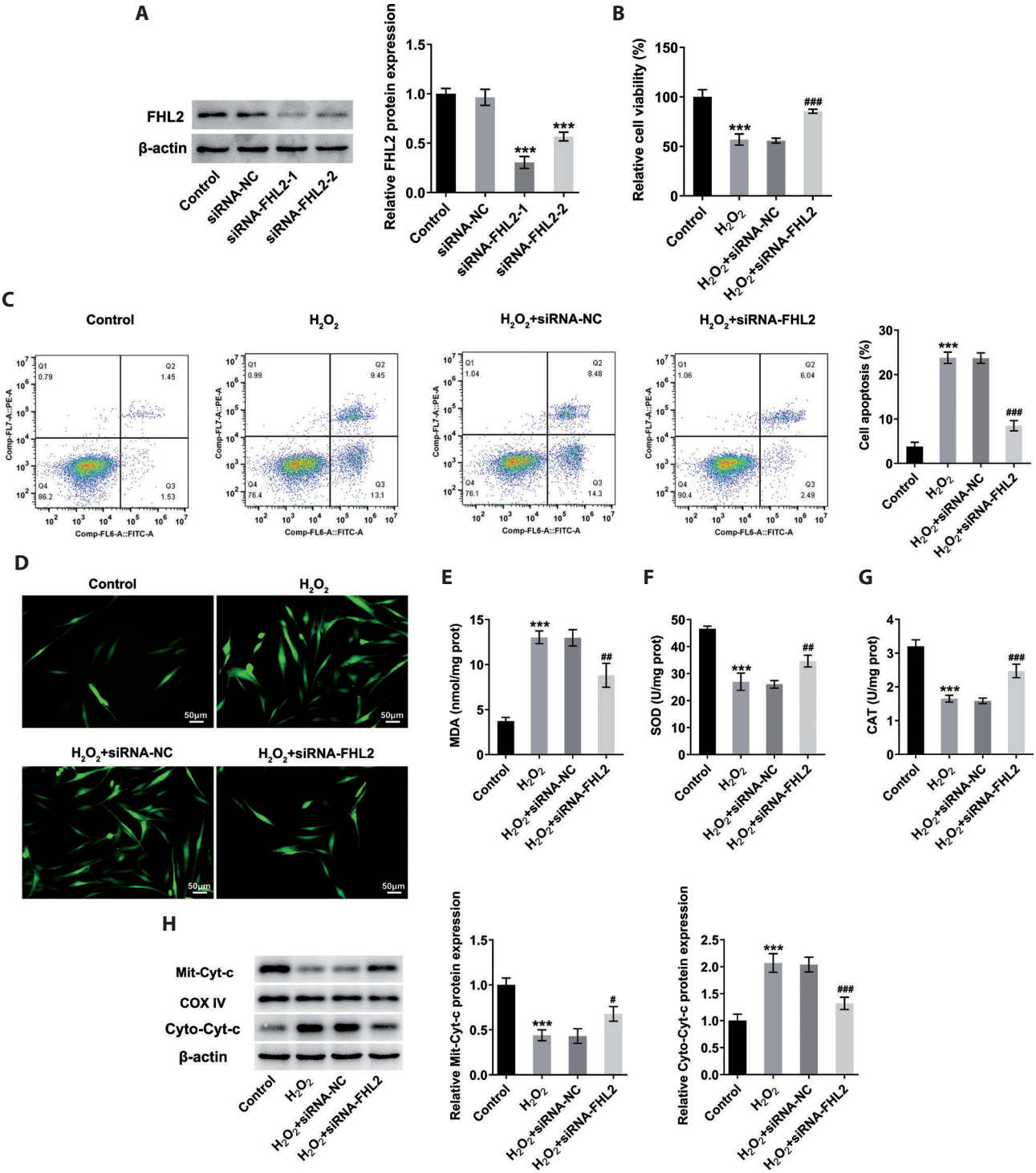
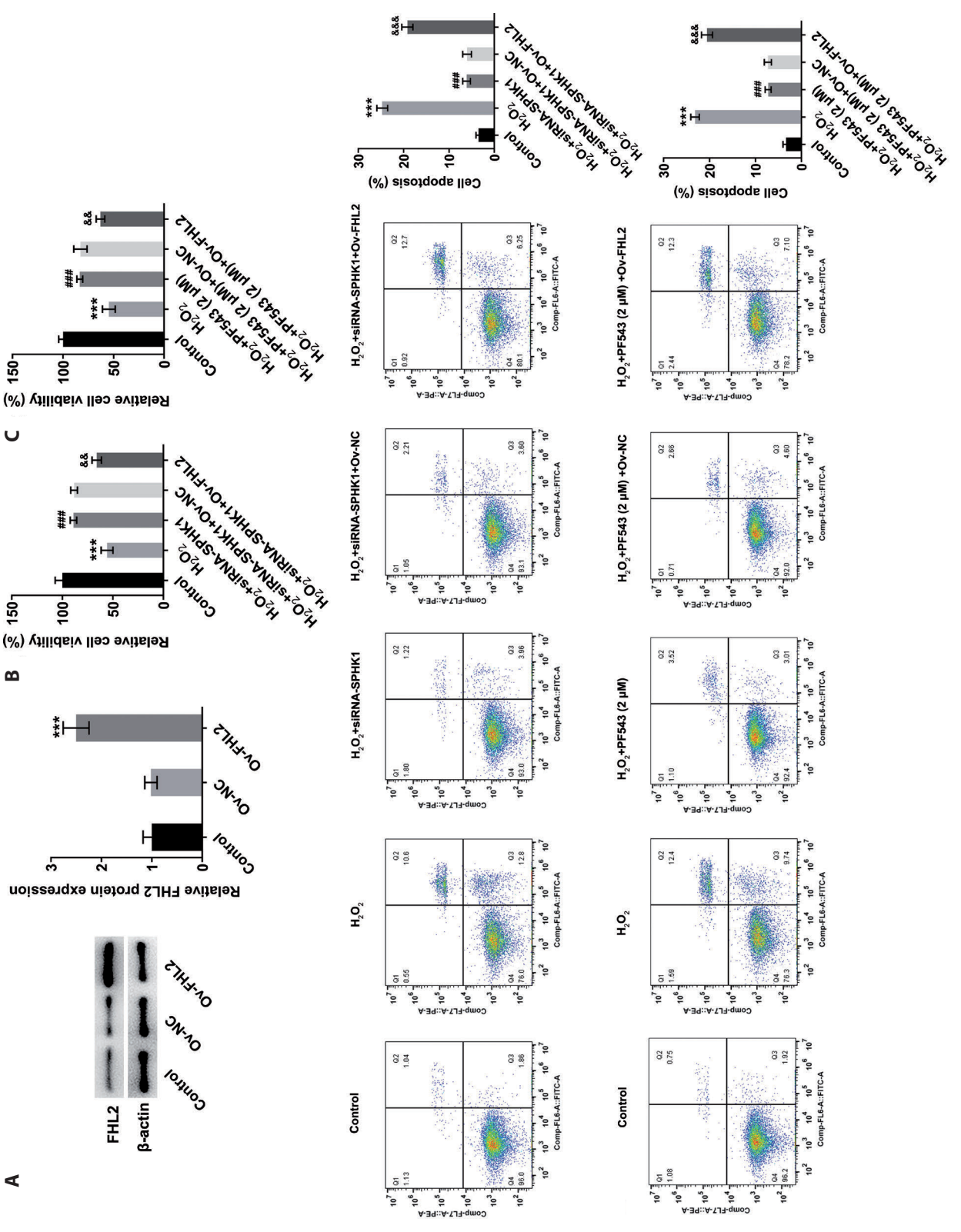


Figure 5. FHL2 depletion suppressed the viability damage, apoptosis and oxidative stress in H_2O_2-challenged PIG1 cells. **A.** FHL2 expression in H_2O_2-challenged PIG1 cells transfected with siRNA-FHL2 was appraised employing Western blot. *** $p < 0.001$ *vs.* siRNA-negative control (NC). **B.** The viability of H_2O_2-challenged PIG1 cells transfected with siRNA-FHL2 was appraised employing CCK-8 assay. **C.** The apoptosis of H_2O_2-challenged PIG1 cells transfected with siRNA-FHL2 was appraised employing flow cytometry. **D.** ROS activity in H_2O_2-challenged PIG1 cells transfected with siRNA-FHL2 was appraised employing DCFH-DA staining. The activities of MDA (**E**), SOD (**F**) and CAT (**G**) in H_2O_2-challenged PIG1 cells transfected with siRNA-FHL2 were appraised employing corresponding assay kits. **H.** The protein contents of Mit-Cyt-c and Cyto-Cyt-c in H_2O_2-challenged PIG1 cells transfected with siRNA-FHL2 were appraised employing Western blot. *** $p < 0.001$ *vs.* Control, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ *vs.* H_2O_2+siRNA-NC.


A
FHL2
β-actin
Control
OV-NC
OV-FHL2
B
Relative FHL2 protein expression
Control
OV-NC
OV-FHL2
***
C
Relative cell viability (%)
Control
H2O2
H2O2+siRNA-SPHK1
H2O2+siRNA-SPHK1+OV-NC
H2O2+siRNA-SPHK1+OV-FHL2
H2O2+PF543 (2 μM)
H2O2+PF543 (2 μM)+OV-NC
H2O2+PF543 (2 μM)+OV-FHL2
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Control
H2O2
H2O2+siRNA-SPHK1
H2O2+siRNA-SPHK1+OV-NC
H2O2+siRNA-SPHK1+OV-FHL2
Cell apoptosis (%)
E
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Cell apoptosis (%)

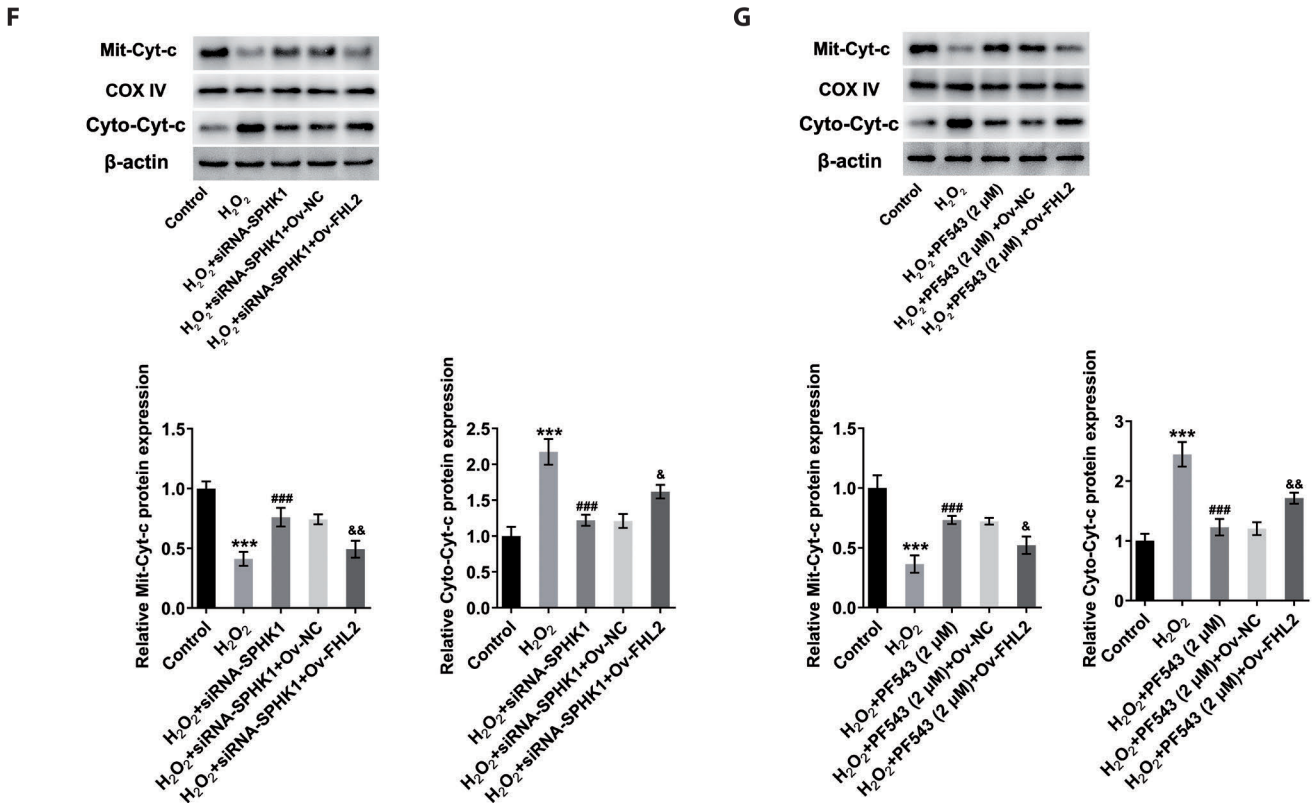

Figure 6. SPHK1 inhibited the viability damage and apoptosis in H_2O_2-challenged PIG1 cells *via* FHL2. **A.** The transfection efficacy of pcDNA3.1 plasmid overexpressing FHL2 (Ov-FHL2) was estimated employing Western blot. *** $p < 0.001$ *vs.* the empty vector (Ov-NC). **B.** The viability of H_2O_2-challenged PIG1 cells co-transfected with siRNA-SPHK1 and Ov-FHL2 was appraised employing CCK-8 assay. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2, && $p < 0.01$ *vs.* H_2O_2+siRNA-SPHK1+Ov-NC. **C.** The viability of H_2O_2-challenged PIG1 cells with PF543 treatment and Ov-FHL2 transfection was appraised employing CCK-8 assay. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2, && $p < 0.01$ *vs.* H_2O_2+PF543 (2μM)+Ov-NC. **D.** The apoptosis of H_2O_2-challenged PIG1 cells co-transfected with siRNA-SPHK1 and Ov-FHL2 was appraised employing flow cytometry. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2, &&& $p < 0.001$ *vs.* H_2O_2+siRNA-SPHK1+Ov-NC. **E.** The apoptosis of H_2O_2-challenged PIG1 cells with PF543 treatment and Ov-FHL2 transfection was appraised employing flow cytometry. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2, &&& $p < 0.001$ *vs.* H_2O_2+PF543 (2μM)+Ov-NC. **F.** The protein contents of Mit-Cyt-c and Cyto-Cyt-c in H_2O_2-challenged PIG1 cells co-transfected with siRNA-SPHK1 and Ov-FHL2 were appraised employing Western blot. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2, & $p < 0.05$ and && $p < 0.01$ *vs.* H_2O_2+siRNA-SPHK1+Ov-NC. **G.** The protein contents of Mit-Cyt-c and Cyto-Cyt-c in H_2O_2-challenged PIG1 cells with PF543 treatment and Ov-FHL2 transfection were appraised employing Western blot. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2, & $p < 0.05$ and && $p < 0.01$ *vs.* H_2O_2+PF543 (2μM)+Ov-NC.

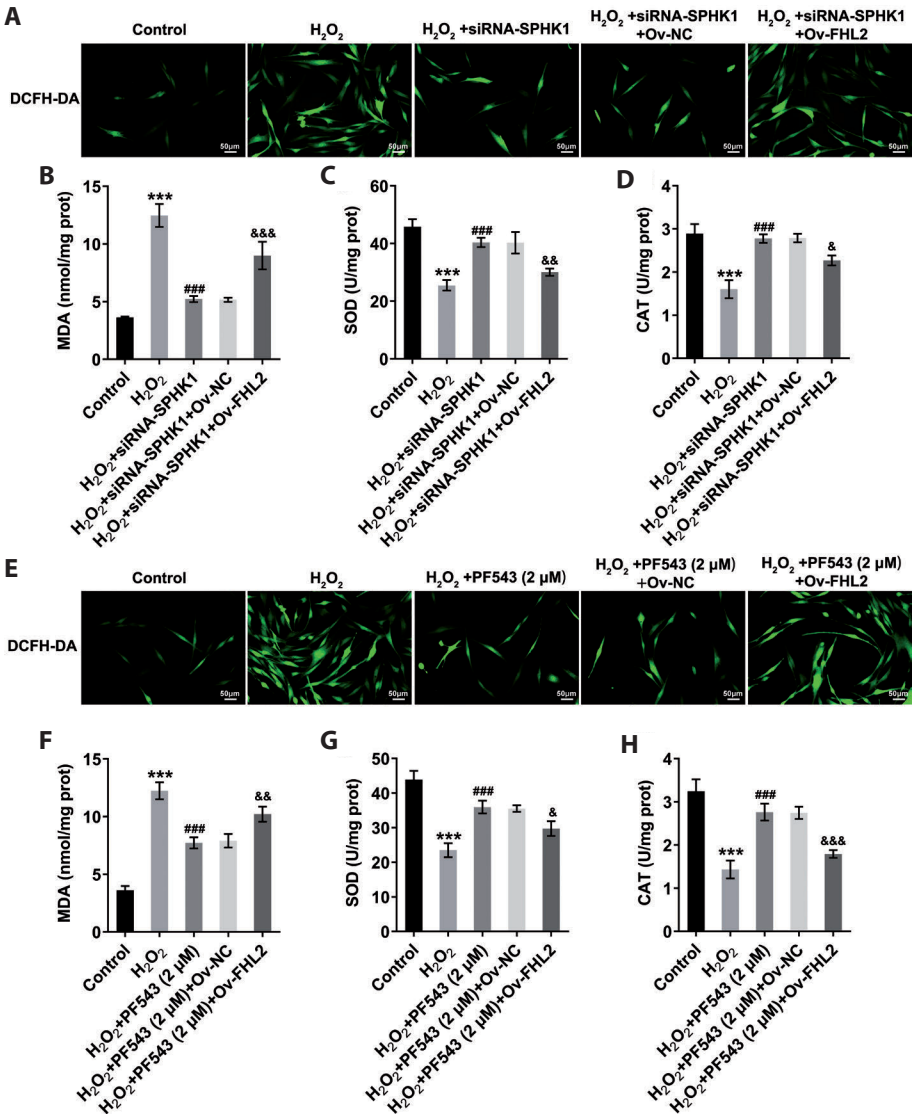

Figure 7. SPHK1 interference alleviated oxidative stress in H_2O_2-challenged PIG1 cells *via* FHL2. **A.** ROS activity in H_2O_2-challenged PIG1 cells co-transfected with siRNA-SPHK1 and pcDNA3.1 plasmid overexpressing FHL2 (Ov-FHL2) was appraised employing DCFH-DA staining. The activities of MDA (**B**), SOD (**C**) and CAT (**D**) in H_2O_2-challenged PIG1 cells co-transfected with siRNA-SPHK1 and Ov-FHL2 were appraised employing corresponding assay kits. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2. & $p < 0.05$, && $p < 0.01$ and &&& $p < 0.001$ *vs.* H_2O_2+siRNA-SPHK1+ the empty vector (Ov-NC). **E.** ROS activity in H_2O_2-challenged PIG1 cells with PF543 treatment and Ov-FHL2 transfection was appraised employing DCFH-DA staining. The activities of MDA (**F**), SOD (**G**) and CAT (**H**) in H_2O_2-challenged PIG1 cells with PF543 treatment and Ov-FHL2 transfection were appraised employing corresponding assay kits. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* H_2O_2. & $p < 0.05$, && $p < 0.01$ and &&& $p < 0.001$ *vs.* H_2O_2+PF543 (2μM)+Ov-NC.

CAT activities in PIG1 cells, which were all reversed after interfering FHL2 (Fig. 5D–G). Results of the Western blot illustrated that H_2O_2 induction reduced Mit-Cyt-c protein content whereas elevated Cyto-Cyt-c protein content in PIG1 cells in comparison with those in Control group, while siRNA-FHL2 imparted opposite impacts on these two proteins, attested by ascended Mit-Cyt-c expression and descended Cyto-Cyt-c expression in H_2O_2+siRNA-FHL2 group (Fig. 5H).

SPHK1 inhibited the viability damage and apoptosis in H_2O_2-challenged PIG1 cells via FHL2

In order to upregulate FHL2 content, Ov-FHL2 was introduced to PIG1 cells and the transfection efficacy was estimated utilizing Western blot. By contrast with the Ov-NC group, FHL2 content was conspicuously increased in Ov-FHL2 group (Fig. 6A). To investigate the mechanism of SPHK1 associated with FHL2 in H_2O_2-insulted PIG1 cells, cells were co-transfected with siRNA-SPHK1 and Ov-FHL2 or treated with SPHK1 inhibitor PF543. Cell were divided into Control, H_2O_2, H_2O_2+siRNA-SPHK1, H_2O_2+PF543 (2 μM), H_2O_2+siRNA-SPHK1+Ov-NC, H_2O_2+PF543 (2μM)+Ov-NC, H_2O_2+siRNA-SPHK1+Ov-FHL2 and H_2O_2+PF543 (2 μM)+Ov-FHL2 groups. Relative to the H_2O_2 group, SPHK1 depletion or SPHK1 inhibitor PF543 revived the viability of PIG1 cells, which was then reduced again after overexpressing FHL2 (Fig. 6B,C). Compared with the H_2O_2+siRNA-SPHK1+Ov-FHL2 or H_2O_2+PF543 (2μM)+Ov-NC group, Ov-FHL2 evidently facilitated PIG1 cell apoptosis (Fig. 6D,E). Furthermore, SPHK1 deficiency or SPHK1 inhibitor PF543 ascended Mit-Cyt-c protein content

whereas descended Cyto-Cyt-c protein content in PIG1 cells with H_2O_2 challenge in contrast to the H_2O_2 group, which were all reversed following the transfection with Ov-FHL2 (Fig. 6F,G).

SPHK1 interference alleviated oxidative stress in H_2O_2-challenged PIG1 cells via FHL2

As Figure 7A–D demonstrated, SPHK1 interference descended ROS and MDA activities whereas ascended SOD and CAT activities in H_2O_2+siRNA-SPHK1 group, which were all reversed after overexpressing FHL2. Similarly, Ov-FHL2 elevated ROS and MDA activities while declined SOD and CAT activities in H_2O_2+PF543 (2μM)+Ov-FHL2 group when compared to the H_2O_2+PF543 (2μM)+Ov-NC group (Fig. 7E–H).

Discussion

Vitiligo is driven by progressive destruction of melanocytes in the skin and excessive H_2O_2 production can be discovered in vitiligo patients at advance stage (Schallreuter et al. 1999). H_2O_2 has been well-documented to play a pivotal role in vitiligo and H_2O_2 elevation can be an indicator for patients with great possibility to develop vitiligo (Song et al. 2008). Additionally, adopting 1.0 mM H_2O_2 to challenge melanocytes is supposed to be a favorable strategy to stimulate oxidative damage incessantly (Jian et al. 2014, 2016). Hence, the present study also adopted this concentration in follow-up studies. Results of this study illuminated that SPHK1 inhibition ameliorated the viability damage, apoptosis and oxidative stress in H_2O_2-challenged PIG1 cells *via* FHL2, which preliminarily disclosed the protective role of SPHK1 in vitiligo and offered a potent therapeutic target in treating vitiligo.

When ROS generation transcends the scavenging capability of the antioxidant system, oxidative stress takes place (Martindale and Holbrook 2002). A growing body of studies have supported that oxidative stress plays a vital role in the pathological alteration in the onset and advancement of vitiligo because of the direct loss or complete damage it brought to functioning melanocytes (Yamamoto et al. 2020). Accumulating studies have manifested that the activities of CAT and SOD in patients suffering from vitiligo are declined (Cui et al. 2019;, Yamamoto et al. 2020). A previous study has attested that SPHK1 upregulation facilitates oxidative stress in high glucose challenged HK-2 cells by elevating MDA activity as well as reducing SOD and CAT activities (Ren et al. 2023). In addition, Zeng et al. (2023) have corroborated that SPHK1 overexpression can exacerbate oxidative stress damage in lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute liver injury. Results of the present study demonstrated that SPHK1 interference or SPHK1 inhibitor PF543 could descend MDA and ROS activities whereas ascend SOD and CAT activities in PIG1 cells insulted by H_2O_2, indicating that SPHK1 exhibits oxidative property and might be a promising therapeutic target for vitiligo.

Apoptosis is a type of cell death, which has been implicated to be critical in the advancement of vitiligo (Tulic et al. 2019). Following ROS attack, melanocytes are vulnerable to apoptosis and autoimmune response is triggered, thus further facilitating cell apoptosis (Colucci et al. 2015; Bergqvist and Ezzedine 2021). In addition, the accumulation of ROS can result in organelle dysfunction, thereby eventually causing melanocyte dysfunction and even melanocyte death (Xuan et al. 2022). It has been well-documented that SPHK1 inhibition can accelerate apoptotic cell death in melanomas (Madhunapantula et al. 2012). Nonetheless, Avni and co-workers have elucidated that the interference or suppression of SPHK1 represses cell apoptosis to alleviate fulminant hepatic failure (Avni et al. 2021). Results of this study illustrated that the silence or suppression of SPHK1 reduced the apoptotic level in PIG1 cells insulted by H_2O_2. It is commonly acknowledged that the distribution of Cyt-c is an indicator of the early trigger of apoptosis (Parra et al. 2008). Results of this study revealed that SPHK1 deficiency or suppression ascended Mit-Cyt-c content whereas descended Cyto-Cyt-c content in H_2O_2-challenged PIG1 cells.

Previous study has illuminated that FHL2 has great potential to be a therapeutic target for vitiligo (Hu et al. 2022). Yan et al. (2023) have put forward that FHL2 has a critical regulatory role in apoptosis. In epithelial ovarian cancer, the depletion of FHL2 can suppress cell growth and induce cell apoptosis (Wang et al. 2020). Additionally, the interference of FHL2 can accelerate neuronal apoptosis in spinal cord injury (Xu et al. 2023). After silencing FHL2 expression, the viability damage, apoptosis and oxidative stress in H_2O_2-challenged PIG1 cells hereby were all alleviated. Intriguingly, hitpredict database predicted that SPHK1 can bind to FHL2 and the binding relationship was presented in HDOCK Sever. Results of this study further confirmed the interaction between SPHK1 and FHL2 and revealed that SPHK1 positively regulated FHL2 in H_2O_2-insulted PIG1 cells.

With the aim of further exploring the mechanism of SPHK1 associated with FHL2 in viability damage, apoptosis and oxidative stress in H_2O_2-insulted PIG1 cells, FHL2 was overexpressed and above functional experiments were implemented again. Results demonstrated that the suppressive impacts of SPHK1 deficiency or inhibition on H_2O_2-challenged viability damage, apoptosis and oxidative stress in PIG1 cells were all offset by FHL2 overexpression, implying that SPHK1 protected PIG1 cells again oxidative stress damage *via* FHL2.

Conclusion

To conclude, this paper revealed that the interference or inhibition of SPHK1 could suppress viability damage, apoptosis and oxidative stress in H_2O_2-insulted PIG1 cells and identified that SPHK1 could bind to FHL2, which firstly disclosed the protective role of FHL2 in vitiligo and offered a prospective therapeutic target in treating vitiligo. However, this study also has some limitations. For instance, the role of SPHK1 in signaling pathways involved in vitiligo hasn't been investigated and the findings of this study haven't been confirmed *in vivo* experiments, both of which will be the research focus in our future studies.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest. The authors declare that they have no competing interests.

Authors' contributions. Kuo-Hsiang Liao conceived the experiments. Kuo-Hsiang Liao and Kuo-Liang Liao performed the experiments. Kuo-Hsiang Liao analyzed the data. Kuo-Hsiang Liao and Kuo-Liang Liao confirmed the authenticity of all the raw data. Both authors have read and approved the final manuscript.

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