ACOT7 protects epidermal stem cells against lipid peroxidation

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Received: 31 January 2022 / Accepted: 10 June 2022 / Published online: 29 August 2022 / Editor: Tetsuji Okamoto © The Author(s) 2022

Abstract

Epidermal stem cells (ESCs) are critical for skin regeneration and repair. Previous studies have shown that ESCs are susceptible to oxidative stress, which in turn leads to lipid peroxidation and affects skin repair. Our study aims to explore how ESCs resist lipid peroxidation. By performing proteomics analysis, we found that the expression of Acyl-CoA thioesterase 7 (ACOT7) was positively correlated with the concentration of transferrin. Overexpression adenovirus vectors of ACOT7 were constructed and transfected into ESCs. Levels of lipid peroxidation by flow cytometry, cell viabilities, and MDA levels were measured. The results revealed that ACOT7 could inhibit lipid peroxidation, reduce the level of malondialdehyde (MDA), and improve the survival rate of ESCs induced by H2O2, Erastin, and RSL3. Our data suggest that ACOT7 has an effect on protecting ESCs against iron-dependent lipid peroxidation.

Keywords Transferrin · ACOT7 · Epidermal stem cells · Lipid peroxidation

Introduction

The skin is the largest organ of the human body, which constantly renews itself to maintain its barrier function (Hennig et al. 2020). Being directly exposed to the environment, the skin is easily affected by ultraviolet radiation, pathogen invasion, and other external threats (Yin et al. 2019). These factors can cause oxidative stress, which in turn affects the function of skin cells. The epidermis, the outermost layer of the skin, is rich in lipids, proteins, and DNA, so it is susceptible to oxidative stress. Epidermal damage can lead to skin aging, poor wound healing, and even skin diseases (Klaunig et al. 2010; An et al. 2013). ESCs are a type of cells located in the basal part of the epidermis, which can differentiate into mature keratinocytes that have an indispensable role during skin healing after trauma (Liao et al. 2014). However, ESCs are easily affected by oxidative stress, which may affect their ability to proliferate and differentiate, thus delaying the wound healing process (Ud-Din et al. 2021).

Iron is a catalyst of oxidative stress. It can bind to different ligands and has the property of electron transfer (Wang et al. 2019). Excessive amounts of intracellular iron may destroy redox homeostasis through the Fenton reaction and catalyze reactive oxygen species (ROS) production, inducing the destruction of macromolecular substances, such as DNA, RNA, protein, and lipid (Fibach 2019). ROS then interacts with polyunsaturated fatty acids on the biomembrane, resulting in cellular structure and function changes, a process also known as lipid peroxidation, which then leads to cell death, i.e., ferroptosis (Tomita et al. 2019). Previous studies suggested that oxidative stress increases with overload iron, which may harm cell functions. This process may be ameliorated by chelating excess intracellular free iron through deferoxamine (Holden and Nair 2019). In addition,
cells may upregulate the expression of some genes, such as ferritin, to relieve the effect. According to previous studies, in keratinocytes and fibroblasts, overexpressed ferritin caused by iron load may significantly inhibit the procedure of lipid peroxidation (Giordani et al. 2000; Zhang et al. 2021). However, the impact of iron load on ESCs needs to be further explored.

ACOT7 is an enzyme that catalyzes the hydrolysis procedure from acyl-CoA to free fatty acid and coenzyme A. In our study, we found that ACOT7 reduces the level of iron-dependent lipid peroxidation. The aim of the present study was to further explore the role of ACOT7 in inhibiting the procedure of lipid peroxidation in ESCs cultured in vitro.

Materials and methods

Animals All animal studies (including the mouse euthanasia procedure) were done in compliance with Shandong University institutional animal care regulations and conducted according to the ARRIVE guidelines and the U.K. Animals (Scientific Procedures) Act 1986 guidelines. A total of 20 specific pathogen-free (SPF) wild-type male C57BL/6 J newborn mice were purchased from the experimental animal center of Shandong University (Permit number: SCXK Lu 20,190,001). All the animals were housed in an environment with a temperature of 22 ± 1°C, relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 h.

Isolation, culture, and identification of ESCs Processes of isolation, culture, and identification of ESCs were conducted according to previous studies (Zhang et al. 2018a, b, 2020). Briefly, 20 mice were used for this experiment. Skin tissues were collected and digested at +4°C overnight in 0.5% Dispase II (Sigma, St. Louis, MO). Then, the epidermis was dissected from the dermis and digested in 0.25% trypsin (Gibco, Crand Island, NY) at +37°C for 7 min; the process of digestion was terminated by adding RMPI 1640 (Thermo Fisher Scientific, Shanghai, China) and 10% FBS (Thermo Fisher Scientific). Samples were then filtrated and centrifuged to obtain cells, which were resuspended in a CnT-Prime culture medium (Celltec, Bern, Switzerland).

Cells were incubated in culture bottles containing type IV collagen (Sigma) at the density of 5 × 10⁵–1 × 10⁶/ml, and they were placed in normoxic incubators containing 5% CO₂ at +37°C for approximately 30 min. After cell adherence, the culture solution was discarded, and cells were gently rinsed with phosphate-buffered saline (PBS) three times. Cells were incubated in a fresh medium in normoxic incubators, and the culture solution was conducted every other day.

Flow cytometry, Western blot analysis, and immunofluorescence analysis were used to identify ESCs. Cytokeratin 14 (CK14), cytokeratin 15 (CK15), p63, and cytokeratin 10 (CK10) were used to differentiate ESCs from keratinocytes.

All experiments were performed with mycoplasma-free cells.

Adenovirus transfection After reaching a 70% confluency, the adenovirus carrying Acot7 (with Flag tag) and the vector adenovirus (without Flag tag) were transfected into ESCs. The multiplicity of infection (MOI) was determined to be 10. The ESCs were divided into the following three groups: (a) experimental group (ESCs were transfected by the adenovirus carrying Acot7); (b) control group (ESCs were transfected by the vector adenovirus); (c) the blank group (ESCs did not receive adenovirus transfection). The adenoviruses were provided by Shanghai Genechem Co., Ltd, Shanghai, China. The culture mediums were changed after the 10-h period of transfection. On the second day, Western blot analysis and immunofluorescence analysis were applied to investigate the transfection efficiency.

Detection of cell proliferation Cell proliferation was analyzed using an IncuCyte S3 Living-Cell Analysis System (Sartorius, Gottingen, Germany). Briefly, ESCs were cultured in 96-well plates at the density of 5 × 10³ cells/plate, and then incubated with CnT-Prime culture medium at 37°C and 5% CO₂ for 48 h. By calculating the area confluence, which was normalized to 0 h, then the cell proliferation curves were drawn.

Detection of cell apoptosis The Annexin V-PE/7-AAD apoptosis analysis kits (BD Biosciences, San Jose, CA) detected apoptosis (Meng et al. 2018). After centrifugation and washing with PBS, ESCs were resuspended using 1 × binding buffer at a density of 1 × 10⁶ cells/ml, and then incubated with PE-conjugated Annexin V and 5 µl of 7-AAD for 15 min at room temperature (25°C) in the dark. Afterwards, samples were detected by CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN). Early apoptotic, late apoptotic, and dead cells can be distinguished on the basis of a double-labeling for Annexin V-PE and 7-AAD.

Detection of cell viability The impacts of H₂O₂, Erastin, and RSL3 on the cell viability of ESCs were measured by the
Detection of the relative levels of lipid peroxidation and Fe^{2+} by flow cytometry The relative intracellular levels of lipid peroxidation and Fe^{2+} were measured by the C11-BODIPY lipid probe (Invitrogen, Carlsbad, CA) and FerroOrange (Dojindo). The cells were gently rinsed with PBS three times, and the adherent cells were digested with trypsin. After digestion, centrifugation was conducted to collect cell pellets, which were resuspended with 100-μl living cell image solution (Thermo Fisher Scientific), and incubated with 0.1 μl C11-BODIPY (Invitrogen, 1:1000) and 0.2 μl FerroOrange (Dojindo, 1:500) in the dark for 30 min. Next, to blow and mix the cells, a 400-μl living cell image solution was added. The wavelength of FerroOrange excitation light was 543 nm, and the wavelength of emission light was 580 nm. Oxidation of the polyunsaturated butadienyl portion of the C11-BODIPY resulted in a shift of the fluorescence emission peak from ~590 to ~510 nm. The relative levels of lipid peroxidation and Fe^{2+} were measured by flow cytometry, which was quantified by the FITC/PE value and the PE value, respectively (Cheloni and Slaveykov 2013; Mei et al. 2020).

Detection of MDA levels The intracellular level of MDA was determined according to the instructions of lipid peroxidation (MDA) assay kit (Abcam, Cambridge, UK). In the lipid peroxidation assay protocol, the MDA in the sample was reacted with thiobarbituric acid (TBA) to generate an MDA-TBA adduct. The MDA-TBA adduct was easily quantified colorimetrically (OD = 532 nm) (Wang et al. 2021).

Protein extraction and Western blot Western blot analysis was performed in order to measure the expression of proteins. The cell lysate was attained with RIPA lysate (Thermo Fisher Scientific) and the protein concentration of the samples was measured with BCA Protein Assay Kit (Thermo Fisher Scientific). Samples (approximately 40 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk for 2 h before being incubated with primary antibodies at 37°C overnight. The primary antibodies including anti-CK10 (1:1000, Abcam, ab76318), anti-CK14 (1:1000, Abcam, ab181595), anti-CK15 (1:1000, Abcam, ab52816), anti-p63 (1:1000, Abcam, ab124762), anti-GAPDH (1:1000, Cell Signaling Technology, #5174, Boston, MA), anti-ACOT7 (1:5000, Proteintech Group, 15,972–1-AP), anti-FLAG (1:500, Sigma, F7425), anti-FTH (1:1000, Invitrogen, 701,934), and anti-FTL (1:1000, Invitrogen, MA5-32,755). The membranes were then rinsed with TBST and incubated with the HRP-conjugated secondary antibody (1:5000, Cell Signaling Technology, #7074) at room temperature for 1 h. The iBright imaging system (Invitrogen) was applied to obtain images, and the ImageJ software was used to quantify the expression of proteins.

Results

ESCs showed high expression of CK14, CK15, and p63 After 2 days of culture, ESCs were observed under a light microscope (Fig. 1A). CK14, CK15, and p63 are markers of ESCs and CK10 is the marker of keratinocytes (Ghadially 2012). Next, Western blot analysis was performed to compare markers expressed on adherent and non-adherent cells. The expression of CK14, CK15, and p63 was significantly higher in the adherent cells than that in the non-adherent cells, and the expression of CK10 was significantly lower in the adherent cells (Fig. 1B). Furthermore, flow cytometry suggested that the positive rates of obtained cells expressing CK14, CK15, and p63 were more than 80%, while the positive rate of CK10 was less than 10% (Fig. 1C). Similar results were observed by immunofluorescence, which suggested that these cells were ESCs (Fig. 1D).

The expression of ACOT7 increases with the rise of iron load in ESCs Next, ESCs were cultured in the medium with 0, 1, 10, 100, or 1000 μg/ml holo-transferrin. Flow cytometry
suggested that with the increase of the concentration of holo-transferrin, there was a significant rise in the level of intracellular Fe\(^{2+}\) (Fig. 2A), while no differences were observed in the level of lipid peroxidation. After the ESCs were stimulated with 500 µM H\(_2\)O\(_2\) for 6 h, no differences in the level of lipid peroxidation were observed (Fig. 2B). Because of the Fenton reaction, we suspected that some genes were upregulated to maintain a stable level of lipid peroxidation. Consequently, we found significant differences in the expression of some proteins, including ACOT7 and Ferritin Light Chain1 (FTL1), through TMT quantitative proteomic analysis (Fig. 2C), which was further confirmed by Western blot (WB). WB analysis suggested that ACOT7, Ferritin Heavy Chain (FTH), and Ferritin Light Chain (FTL) upregulation was positively correlated with the concentration of transferrin (Fig. 2D).

**The overexpression of ACOT7 has no impact on the proliferation and apoptosis of ESCs** The experimental group (ESCs were transfected by the adenovirus carrying Acot7) and the control group (ESCs were transfected by the vector adenovirus) were differentiated using the Flag tag. Western blot analysis and immunofluorescence analysis suggested that ESCs in the experimental group expressed a high Flag level

Figure 1. ESCs showed high expression of CK14, CK15, and p63. (A) ESCs cultured for 2 d. Scale bar= 50 µm. (B) WB and quantification showed CK14, CK15, p63, and CK10 expression levels between adherent and non-adherent cells. (C) Flow cytometry and quantification of positive rates of obtained cells expressing CK14, CK15, p63, and CK10. (D) Immunofluorescence identification of obtained cells expressing CK14, CK15, p63, and CK10. Scale bar=20 µm. Statistics: Data were obtained in triplicate experiments and are shown as the mean ± SD; unpaired t-test. *P < 0.05, **P < 0.01.
compared to the control group (Fig. 3A and B). The results indicated the efficiency of virus transfection in the present study. Moreover, the cell proliferation experiment and flow cytometry result showed no differences in the cell proliferation curve and the apoptosis ratio between the two groups (Fig. 3C and D).

The overexpression of ACOT7 decreases the levels of MDA and lipid peroxidation To verify the function of ACOT7, we divided the cells into the following three groups: the experimental group, the control group, and the blank group. There were no differences in the level of lipid peroxidation measured by flow cytometry without oxidative stress. After being incubated with \( \text{H}_2\text{O}_2 \), Erastin, and RSL3 for 6 h, the lipid peroxidation level was significantly lower in the experimental group than that in the control and blank groups (Fig. 4A). We also found that under the condition of the same oxidative stress, the MDA level was significantly lower in the experimental group than that in the control and blank groups (Fig. 4B). Besides, the result of the CCK-8 experiment showed that the cell viability of ESCs was significantly higher in the experimental group than that in the control and blank groups (Fig. 4C).

Figure 2. The expression of ACOT7 increases with the rise of iron load in ESCs. (A) Flow cytometry quantification of cellular Fe\(^{2+}\) level in the medium with 0, 1, 10, 100, or 1000 µg/ml holo-transferrin. (B) Flow cytometry quantification of cellular lipid peroxidation level with no irritants and 500 µM \( \text{H}_2\text{O}_2 \). (C) ESCs cultured in the medium with 100 µg/ml holo-transferrin and no holo-transferrin; ACOT7 and FTL1 showed differences through TMT quantitative proteomic analysis. (D) WB and quantification showed ACOT7, FTL, and FTH expression levels in the medium with 0, 1, 10, 100, or 1000 µg/ml holo-transferrin. Statistics: Data were obtained in triplicate experiments and are shown as the mean±SD; unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.
Iron plays a key role in oxidative stress and light-induced skin damage. It is essential for cells to regulate the balance of oxidative stress (Wang et al. 2014). Given continuous exposure to outside environment, how the skin cells resist oxidative stress is very important (Balic and Mokos 2019). Our study found that ACOT7, which may increase with the rising expression of iron load, has a protective effect on lipid peroxidation induced by H2O2, Erastin, and RSL3 in ESCs. Previous studies showed that free iron induces the production of free radicals, contributing to an increase of the level of lipid peroxidation induced by H2O2, Erastin, and RSL3 in ESCs. Autophagy also regulates intracellular free iron through ferritin degradation (Jacomim et al. 2019). Previous studies have shown that ACOT7 might regulate the metabolism of neuronal fatty acid and prevent neurotoxicity (Ellis et al. 2013). As ACOT7 is closely related to lipid metabolism, we discovered that ACOT7 might inhibit lipid peroxidation in ESCs. H2O2, Erastin, and RSL3 were applied to induce lipid peroxidation in ESCs because Erastin and RSL3 are classical inducers of ferroptosis (Shintoku et al. 2017). Erastin impairs cellular antioxidant defenses, facilitating toxic ROS accumulation by inhibiting system xc−, cysteine-dependent glutathione (GSH) synthesis, and inhibiting the trans-plasma membrane cysteine redox shuttle (Banjac et al. 2008; Dixon et al. 2012). RSL3 can directly decrease the expression of glutathione peroxidase 4 (GPX4), elevating the ROS level in cells (Sui et al. 2018).

We found that the overexpression of ACOT7 may decrease MDA and lipid peroxidation levels, resulting in a higher survival rate of ESCs. We also found that the overexpression of ACOT7 had no impact on cell proliferation and apoptosis, which suggests that ACOT7 may improve the cellular survival rate by inhibiting ferroptosis.

It is not clear how ACOT7 contributes to protecting ESCs against lipid peroxidation. Previous studies have shown that

![Figure 3](image-url)

Figure 3. The overexpression of ACOT7 has no impact on the proliferation and apoptosis of ESCs. (A, B) WB and immunofluorescence showed Flag expression levels between experimental group (ESCs were transfected by the adenovirus carrying Acot7) and the control group (ESCs were transfected by the vector adenovirus). Scale bar = 20 µm. (C) IncuCyte S3 Living-Cell Analysis System measured cell proliferation. (D) Flow cytometry detected cell apoptosis between experimental group and the control group. Statistics: Data were obtained in triplicate experiments and are shown as the mean ± SD; unpaired t-test. ns, not significant.
ACOT7 might interact with a series of long-chain acyl-CoA enzymes, especially arachidonoyl-CoA (AA-CoA), an important precursor of AA-phosphatidylethanolamine (AA-PE) that forms the cell membrane. Besides, AA-PE is likely to be attacked by oxidation, which is a signal of ferroptosis (Doll et al. 2017; Kagan et al. 2017). However, further research is needed to explore whether ACOT7 may regulate the level of AA-PE on the cell membrane to execute its function of inhibiting lipid peroxidation.

In conclusion, we found that ACOT7 may protect cells against lipid peroxidation and improve the cellular survival rate for the first time, which provides us with a new method to inhibit the damage of ESCs caused by lipid peroxidation under oxidative stress, a new idea to prevent ferroptosis and a new direction to explore how ACOT7 exercises its functions.

**Abbreviations**

ESCs: Epidermal stem cells; ACOT7: Acyl-CoA thioesterase 7; MDA: Malondialdehyde; ROS: Reactive oxygen species; SPF: Specific pathogen-free; PBS: Phosphate-buffered saline; CK14: Cytokeratin 14; CK15: Cytokeratin 15; CK10: Cytokeratin 10; CCK-8: Cell Counting Kit-8; FTH: Ferritin Heavy Chain; FTL: Ferritin Light Chain; AA-CoA: Arachidonoyl-CoA; AA-PE: AA-phosphatidylethanolamine

**Author contributions**

Conceptualization: GZ, SY, YW; data curation: GZ, SY, JM, ZW, GC; formal analysis: GZ, SY, JM, ZW; funding acquisition: YW; Investigation: GZ, SY, RS, JM, GC, ZW; methodology: GZ, SY, ZW; project administration: GZ, SY, CL, YW; Resources:
Funding This study was supported by the National Natural Science Foundation of China (81972947), Natural Science Foundation of Shandong Province of China (Major Basic Research Program) (ZR2019ZD38), “Academic Elevation Program” of Shandong First Medical University (Shandong Academy of Medical Sciences) (2019LJ005), and Jinan Clinical Research Center for Tissue Engineering Skin Regeneration and Wound Repair.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Consent to participate Not applicable.

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