Received: 16 April 2023 | Revised: 30 July 2023 | Accepted: 4 September 2023

DOI: 10.1111/exd.14970

RESEARCH ARTICLE

Experimental Dermatology WILEY

Exosomes derived from human dermal fibroblasts (HDFn-Ex) alleviate DNCB-induced atopic dermatitis (AD) via PPARα

You Na Jang[1] | Jung Ok Lee[1] | Jung Min Lee[1] | A. Yeon Park[1,2] | Yu Jin Kim[1,2] | Su Young Kim[1,2] | Joon Seok[1,2] | Kwang Ho Yoo[3] | Beom Joon Kim[1,2]

[1]Department of Medicine, Graduate School, Chung-Ang University, Seoul, South Korea

[2]Department of Dermatology, Chung-Ang University College of Medicine, Seoul, South Korea

[3]Department of Dermatology, Chung-Ang University Gwang-Myeong Hospital, Chung-Ang University College of Medicine, Seoul, South Korea

Correspondence
Beom Joon Kim and Kwang Ho Yoo, Department of Medicine, Graduate School, Chung-Ang University, Seoul, South Korea.
Email: beomjoon74@gmail.com and psyfan9077@naver.com

Funding information
Chung-Ang University Research Scholarship Grants; National Research Foundation of Korea, Grant/Award Number: 2021R1G1A1007827

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Skin barrier dysfunction is the initial step in the development of AD. Recently, exosomes have been considered as potential cell-free medicine for skin defects such as aging, psoriasis and wounds. The aim of this study was to investigate the effects of human dermal fibroblast-neonatal-derived exosome (HDFn-Ex) on AD. HDFn-Ex increased the expression of peroxisome proliferator activated receptor α (PPARα) and alleviated the 1-chloro-2,4-dinitrobenzene (DNCB)-mediated downregulation of filaggrin, involucrin, loricrin, hyaluronic acid synthase 1 (HAS1) and HAS2 in human keratinocyte HaCaT cells. However, these effects were inhibited by the PPARα antagonist GW6471. In the artificial skin model, HDFn-Ex significantly inhibited DNCB-induced epidermal hyperplasia and the decrease in filaggrin and HAS1 levels via a PPARα. In the DNCB-induced AD-like mouse model, HDFn-Ex administration reduced epidermis thickening and mast cell infiltration into the dermis compared to DNCB treatment. Moreover, the decreases in PPARα, filaggrin and HAS1 expression, as well as the increases in IgE and IL4 levels induced by DNCB treatment were reversed by HDFn-Ex. These effects were blocked by pre-treatment with GW6471. Furthermore, HDFn-Ex exhibited an anti-inflammatory effect by inhibiting the DNCB-induced increases in IκBα phosphorylation and TNF-α expression. Collectively, HDFn-Ex exhibited a protective effect on AD. Notably, these effects were regulated by PPARα. Based on our results, we suggest that HDFn-Ex is a potential candidate for treating AD by recovering skin barrier dysfunction and exhibiting anti-inflammatory activity.

KEYWORDS
atopic dermatitis, dermal fibroblast, exosomes, filaggrin, keratinocyte, PPARα, skin barrier

You Na Jang and Jung Ok Lee contributed equally to this work.

1 | INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with symptoms including eczematous lesions, impaired epidermal barrier, pruritus and dry skin.[1] AD is caused by a combination of complex interactions between genetic and environmental factors, the skin barrier and immune dysregulation.[2] Regarding the role of skin barrier in AD, a permeability barrier protects against extensive water loss and prevents the entry of harmful substances like irritants, allergens and microorganisms into the skin.[3] The skin is composed of three main layers: epidermis, dermis and the subcutaneous layer.[4] Among them, the epidermis is the outermost layer and plays an essential role in protection, as it forms the body's first barrier.[5] In AD, skin barrier damage occurs first in non-lesional skin,[3] indicating that disruption of skin barrier is an early trigger of AD and the improvement of damaged skin barrier is a key factor to threat AD.

The peroxisome proliferator-activated receptors (PPARs) govern permeability barrier homeostasis, epidermal differentiation, lipid synthesis and inflammation.[6,7] There are three known PPAR isotypes: PPAR-α, PPAR-β/δ and PPAR-γ.[6] In the skin, PPAR-α activators induce keratinocyte differentiation and epidermal lipid synthesis, including ceramide production,[8] thereby accelerating the morphologic and functional maturation of the epidermal permeability barrier. A hydroxy stearic acid derived from plants promotes barrier repair by significantly increasing filaggrin and transglutaminase-1(TGM1) after treatment.[9] Wy14643, a PPAR-α agonist, significantly increases ceramide and cholesterol ester levels in reconstructed epidermis.[10]

Exosomes are derived from the multivesicular body (MVB), an organelle within the cell, and are secreted from the cell.[11,12] They play a potential messenger role and contain various phospholipids and proteins such as receptor proteins, enzymes, heat shock protein family, glycosylphosphatidylinositol (GPI), as well as genetic materials such as DNA and RNA derived from the cell of origin.[13,14] Due to their characteristics, they can transmit various information to other cells or autologous cells to regulate cell proliferation and differentiation, apoptosis and angiogenesis.[15,16] Accordingly, the use of exosomes has substantially increased for the diagnosis and treatment of various pathologies.[17]

Exosomes secreted by autologous dermal fibroblasts accelerate diabetic cutaneous wound healing by activating the Akt/β-catenin pathway.[18] Topical application of human embryonic mesenchymal stem cell (MSC)-derived exosomes also reduces the critical psoriatic cytokines IL-17 and IL-23 in the skin of a mouse model of psoriasis.[19] Furthermore, exosomes derived from adipose tissue-derived mesenchymal stem cells (ASCs) downregulate the expression of inflammatory cytokines, such as IL-4, IL-23, IL-31 and TNF-α, in an AD mouse model.[20]

To date, topical corticosteroids (CS; dexamethasone), topical calcineurin inhibitors (TCI; cyclosporine), anti-histamines, and Janus kinase (JAK) inhibitors (upadacitinib and abrocitinib) are used as AD therapeutics.[21] Although these drugs may relieve certain symptoms, long-term use of topical steroids may lead to skin thinning with subsequent bleeding.[22] Thus, new biological drugs with high efficacy and safety are in demand. The key benefits of exosomes are their high stability, non-immune rejection, and the direct stimulation of target cells.[23] Although the therapeutic effects of exosomes on AD have been demonstrated,[20] exosomes derived from human neonatal dermal fibroblasts (HDFn-Ex) have not yet been reported. Therefore, we investigated the effects of HDFn-Ex on skin barrier dysfunction in AD-like in vivo and in vitro models.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

HaCaT cells, human keratinocyte cell line, were purchased from ATCC (Manassas, VA, USA), and all the reagents used for cell culture were purchased from WELGENE Inc. (Gyeongsangbuk-do, Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in an incubator at 37°C with 5% CO_2. HaCaT cells were seeded at 2×10^6 cells/well in a 6-well plate and were treated with 5 or 10 μM 1-chloro-2,4-dinitrobenzene (DNCB; Sigma-Aldrich, St Louis, MO, USA), HDFn-Ex (5×10^5 or 1×10^7/mL), and 20 μM GW6471, a PPARα antagonist (Sigma-Aldrich), for 1 or 24 h, respectively.

2.2 | Isolation and characterization of exosomes

Human dermal fibroblast-neonatal (HDFn) cells were purchased from Gibco (Waltham, MD, USA). Exosomes used in this study were isolated from the supernatant of HDFn cells. To this end, HDFn was incubated in serum-free DMEM until the cells reached 70%–80% confluence. The conditioned medium (CM) was collected and centrifuged at 300 × *g* for 10 min and 2000 × *g* for 20 min, followed by filtration to remove cells and cellular debris. The clarified supernatant was collected and concentrated using a sterile-membrane T-series cassette (Pall Life Sciences, Washington, NY, USA) with tangential flow filtration (TFF). The mixture was then centrifuged at 100000 × *g* for 3 h. The HDFn-Ex morphology was examined using a field-emission scanning electron microscope (FE-SEM) (Sigma HD, Carl Zeiss Meditec AG, Jena, Germany). The size distribution was determined using nanoparticle tracking analysis on ZetaView (Particle Metrix, Diessen, Germany). Two exosome markers, CD63 and ALIX, were used, and calnexin was utilized as a negative marker.

2.3 | Cell viability

HaCaT cells were cultured in 96-well plates at a density of 5×10^3 cells/well and were exposed to HDFn-Ex at concentrations ranging

from 5×10^5 to 5×10^7 particles/mL. After 24 h, the cells were incubated in a medium containing WST-8 solution (Biomax, Seoul, Korea) for 2 h at 37°C with 5% CO_2. The absorbance was measured at 450 nm using a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.4 | Western blot analysis

Cells were lysed in PRO-PREP™ (iNtRON Biotechnology, Seongnam, Korea), and the protein content was quantified using the Bradford reagent (Sigma-Aldrich). Equal amounts of protein samples were separated using 10% sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis and transferred to nitrocellulose membranes. After blocking in 5% skim milk solution, the membranes were probed with specific primary antibodies. Antibodies against p38, p-p38(Thr^{180}/Tyr^{182}), JNK, p-JNK(Thr^{183}/Tyr^{182}), ERK, p-ERK (Thr^{202}/Tyr^{204}), IκBα and p-IκBα(Ser^{32}/^{36}) were obtained from Cell Signaling Technology (Danvers, MA, USA); those against PPARβ, HAS2, TNF-α and β-actin were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA); those against CD63, ALIX, Calnexin, PPARα, PPARγ, involucrin, loricrin and HAS1 were purchased from Abcam (Cambridge, UK); and antibodies against filaggrin were obtained from Thermo Fisher Scientific (Rockford, IL, USA).

After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG secondary antibodies obtained from Vector Laboratories (Burlingame, CA, USA). The protein bands were then visualized using an enhanced chemiluminescence detection system (Amersham International PLC, Buckinghamshire, UK). The results were analysed using the Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5 | Cellular reactive oxygen species (ROS) assay

ROS production was evaluated using the cellular ROS detection Assay Kit (ab113851, Abcam) according to the manufacturer's protocol. Cells pre-loaded with 20 μM 2′,7′-dichlorofluorescin diacetate (DCFDA) solution for 30 min were exposed to DNCB, HDFn-Ex and GW6471. Cells were then observed immediately under a fluorescent microscope (Leica Microsystems, Heidelberg, Germany).

2.6 | Culture and treatments of reconstructed three-dimensional (3D) human skin

Reconstructed 3D human skin samples (Neoderm-ED) were purchased from Tego-Science (Seoul, Korea) and treated with DNCB, HDFn-Ex or GW6471 according to the experimental conditions for 24 h. Thereafter, the skin tissue was incubated at 37°C with 5% CO_2. Artificial skin tissue was fixed in 4% formalin for haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC).

2.7 | Sensitization and treatment of AD mice

A total of 25 female SKH-1 hairless mice (age, 6 weeks; weight, 17–20 g; $n=5$ per group) were purchased from Orient Bio (Orient Bio Inc., Seongnam, Korea). All animal experiments were conducted in accordance with the principles of laboratory animal care of the National Institutes of Health (NIH, Bethesda, Maryland, USA) and with the approval of the Ethics Committee for Laboratory Animals at Chung-Ang University. The mice were randomly divided into five groups, with five mice in each group: Normal (Group 1), DNCB only (Group 2), DNCB + Dexamethasone (Group 3), DNCB + HDFn-Ex (Group 4), and DNCB + HDFn-Ex + GW6471 (Group 5). After resting for 1 week, the mice were sensitized on the back skin with 200 μL of 2% DNCB solution, which was dissolved in a 3:1 mixture of acetone and olive oil. Starting from Day 7 after the initial sensitization, the back skin of mice was challenged with 200 μL of 0.5% DNCB mixture, which was repeatedly applied every 2 days for 3 weeks. Equal volumes of acetone and olive oil mixture were used as controls in the normal group. Next, for 2 weeks, GW6471 was applied topically before the DNCB treatment, and then DNCB was applied to the dorsal skin. Dexamethasone was topically applied, and HDFn-Ex was administrated through intraperitoneal injection after treatment with DNCB.

2.8 | Measurement of dermatitis severity

The dorsal skin was photographed in proximity using a digital single-lens reflex camera (D5200; Nikon, Tokyo, Japan). According to the method of previously described,[24] the severity of AD-like dorsal skin lesions was assessed by dermatitis score at the end of the experimental period. The dorsal skin severity scores were recorded for AD mice based on four skin symptoms (excoriation, scaling, oedema, erythema). The scoring range indicators were 0 (none), 1 (mild), 2 (moderate) and 3 (severe). Clinical skin score was defined as the sum of the individual scores with a maximum score of 12.

2.9 | Histological analysis

Paraffin-embedded tissue sections were deparaffinized and stained according to a previously published protocol.[25] To evaluate epidermal thickening, tissue slides were stained with H&E. mast cells in the skin were stained with toluidine blue (TB). For staining of IHC, the slides were blocked with a normal serum solution. Subsequently, sections from the same paraffin block were incubated with primary antibodies, followed by the addition of secondary antibodies. The slides were washed with Tris-buffered saline with 0.1% Tween® 20 Detergent (TBS-T) and incubated with a two-component

high-sensitivity 3,3-diaminobenzidine (DAB) chromogenic substrate (Vector Laboratories). After washing with tap water, the slides were counterstained with haematoxylin. The slides were washed with tap water again, air-dried, mounted, covered with a cover glass and examined under a microscope (3DHISTECH, Budapest, Hungary).

2.10 | Transepidermal water loss (TEWL) and corneometer

TEWL (g/m^2h) and hydration (arbitrary units, A.U.) in the stratum corneum (SC) were measured using a Tewameter (Courage Khazaka Electronic GmbH, Cologne, Germany) and a Corneometer® CM 825(Courage Khazaka Electronic GmbH), respectively, at the end of the experiment. The measurement site was maintained at an indoor temperature of 22–24°C and a humidity of 50%–60%. The measurement results were recorded three times (excluding the initial value), and the average value was determined.

2.11 | Measurement of IgE and IL4

The test animals were sacrificed, and whole blood was collected at the end of the experiment. The blood samples were centrifuged at $3000\times g$ for 10 min at 4°C, and the supernatant was collected. The total amount of IgE and IL4 in the serum was measured using an IgE, IL4 mouse uncoated enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Waltham, USA) per the manufacturer's instructions. All factors were measured using a SpectraMax 190 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

2.12 | Statistical analyses

The data were analysed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA), and all quantitative data are presented as the mean ± SD. Experimental data were obtained from at least three independent experiments. One-way ANOVA was used for comparisons of multiple groups. Statistically significant differences were indicated as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3 | RESULTS AND DISCUSSIONS

3.1 | Isolation and characterization of HDFn-Ex

HDFn-Ex isolation was performed using ultracentrifugation (UC) and TFF methods (Figure 1A). HDFn-Ex exhibited a spherical morphology with a diameter of 100–300 nm (Figure 1B). In order to confirm the production yield of isolated exosomes, the number and size of total particles were quantified using NTA. The mean size and abundance of exosomes were 209.1 ± 107 nm and $3.17\times10^{10}\pm1.53\times10^{9}$ per mL, respectively (Figure 1C). Two exosome markers, CD63 and ALIX, were abundant in HDFn-Ex samples, but no calnexin, the negative protein marker of exosomes, was detected (Figure 1D). These results indicated that highly pure samples of HDFn-Ex were successfully obtained. To investigate the cytotoxicity of HDFn-Ex in HaCaT cells, we performed a WST-8 assay. As shown in Figure. 1E, no cytotoxicity was observed at concentrations up to 5×10^{7} particles/mL.

3.2 | Only PPARα expression is increased by HDFn-Ex

To determine whether HDFn-Ex affects the expression of PPARs in HaCaT cells, we assessed their levels using western blotting after HDFn-Ex treatment. Among the PPARs, only PPARα exhibited increased protein levels in a dose-dependent manner. The levels of the other isotypes, PPARβ and PPARγ, were not affected (Figure 2A). The DNCB-induced AD-like in vitro model exhibited decreased PPARα expression. In contrast, co-treatment with DNCB and HDFn-Ex suppressed DNCB-mediated PPARα downregulation (Figure 2B,C).

3.3 | HDFn-Ex inhibits DNCB-mediated downregulation of filaggrin, involucrin, and loricrin via PPARα

DNCB-treated HaCaT cells showed strong fluorescence, indicating excessive ROS generation. By contrast, co-treatment with HDFn-Ex and DNCB reduced the cellular ROS levels, and this effect was blocked by GW6471, a PPARα antagonist, indicating that HDFn-Ex suppressed DNCB-mediated ROS production via PPARα (Figure 3A). HDFn-Ex suppressed the DNCB-mediated phosphorylation of p38(Thr^{180}/Tyr^{182}), JNK(Thr^{183}/Tyr^{185}) and ERK(Thr^{202}/Tyr^{204}); however, pre-treatment with GW6471 abrogated these beneficial effects of HDFn-Ex (Figure 3B). The expression levels of filaggrin, involucrin, and loricrin were reduced by DNCB treatment. In contrast, pre-treatment with HDFn-Ex inhibited DNCB-mediated decreases in filaggrin, involucrin and loricrin levels, and this inhibitory effect was abrogated by GW6471, suggesting that the protective effect of HDFn-Ex on skin barrier is mediated on PPARα. Dry skin is a common symptom found in AD patients.[26] The key molecule involved in skin moisture is hyaluronan or hyaluronic acid (HA).[27] Therefore, we examined the hydration effects of HDFn-Ex in DNCB-treated HaCaT cells. The levels of HAS1 and HAS2 decreased, but HDFn-Ex inhibited DNCB-mediated downregulation of HAS1 and HAS2. Notably, this effect was reversed by GW6471 (Figure 3C). In HaCaT cells with downregulation of PPARα using PPARα siRNA, we also confirmed the HDFn-Ex did not reverse DNCB-induced decrease in these protein levels. (Figure S1). These results demonstrate that HDFn-Ex protects skin hydration by regulating PPARα. AD increases the thickness of the SC due to hyperkeratosis.[28] DNCB led to the thickening of the epidermis. In contrast, the epidermis layer co-treated with HDFn-Ex and DNCB was thinner compared to DNCB-only-treated tissues, indicating that HDFn-Ex suppressed hyperkeratosis. Interestingly, this

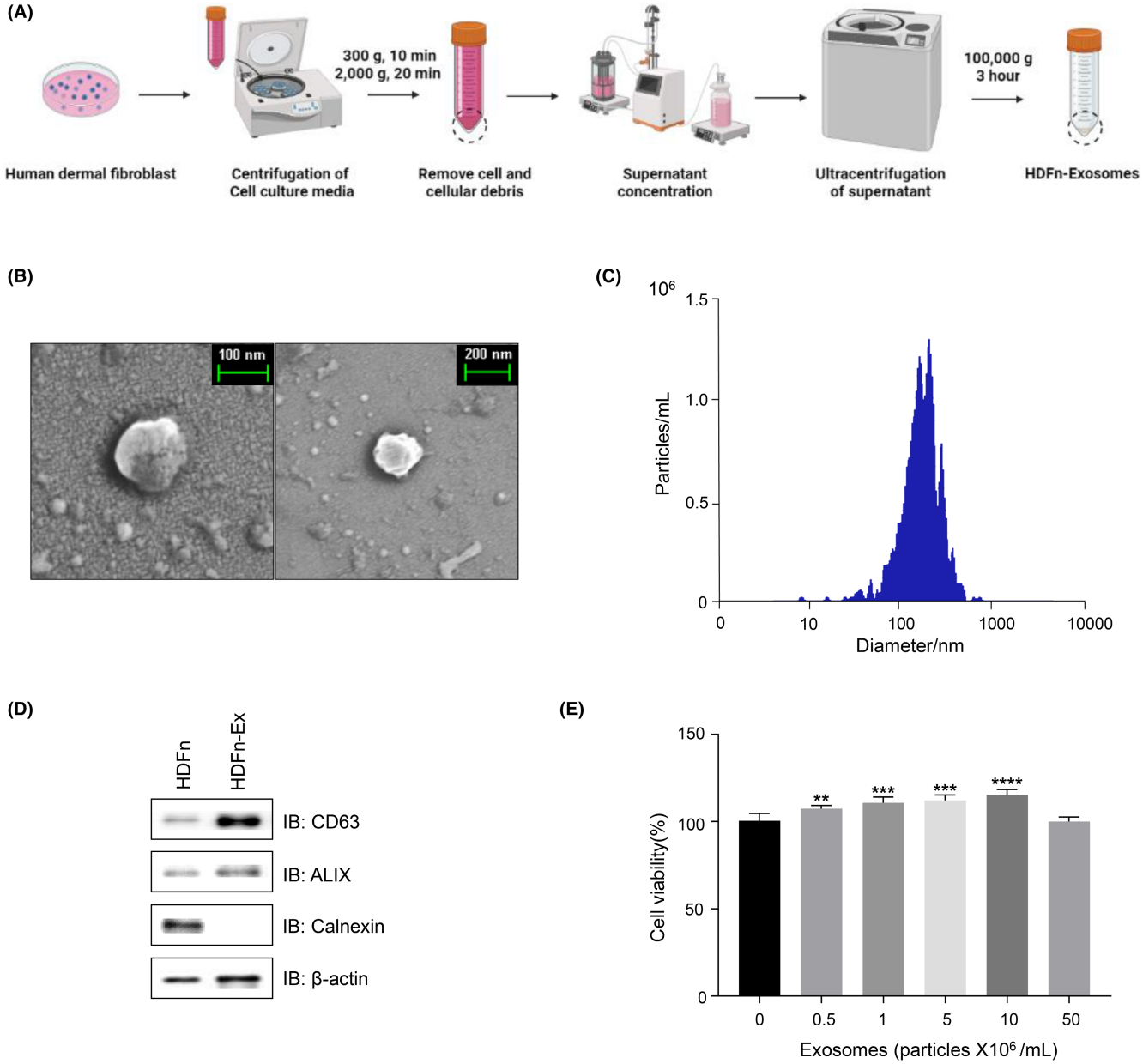

FIGURE 1 Isolation and characterization of HDFn-Ex.(A) Scheme of exosome isolation methods. (B) Image of HDFn-Ex obtained using a FE-SEM. Scale bar, 200 μm. (C) Histogram of particle concentration and size distribution of HDFn-Ex measured using NTA. (D) Expression levels of CD63, ALIX and calnexin after treatment with HDFn-Ex for 24 h. (E) HaCaT cells were treated with HDFn-Ex (5×10^5 to 10^7 particles/mL) for 24 h, and WST-8 cell viability assay was performed. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared with the controls (unpaired *t*-test).

effect was inhibited by GW6471 treatment (Figure 3D,H). Moreover, HDFn-Ex blocked DNCB-induced decreases in PPARα (Figure 3E,I), Filaggrin (Figure 3F,J) and HAS1 (Figure 3G,K) levels, and these effects were suppressed by GW6471 treatment.

3.4 | HDFn-Ex improves skin lesions in DNCB-induced AD-like hairless mouse model

We established an AD-like lesion model in SKH-1 mice induced by DNCB and investigated the role of HDFn-Ex in AD-like mice using dexamethasone as a positive control. The experimental schedule is presented in Figure 4A. Photographs of the mouse dorsal skin (Figure 4B) and SCORAD scores (Figure 4G) showed that HDFn-Ex significantly alleviated the clinical symptoms on the dorsal skin. However, the effect of HDFn-Ex was inhibited by GW6471 treatment. DNCB increases the thickness of the SC due to hyperkeratosis. Compared with the Group 2 (DNCB only), the epidermal thickness of the Group 3 (DNCB + Dexamethasone) and Group 4 (DNCB + HDFn-E) significantly decreased. This HDFn-Ex effect was inhibited by GW6471(Figure 4C,H). Transepidermal water loss (TEWL) in the Group 2 (DNCB only) increased compared with the Group 1

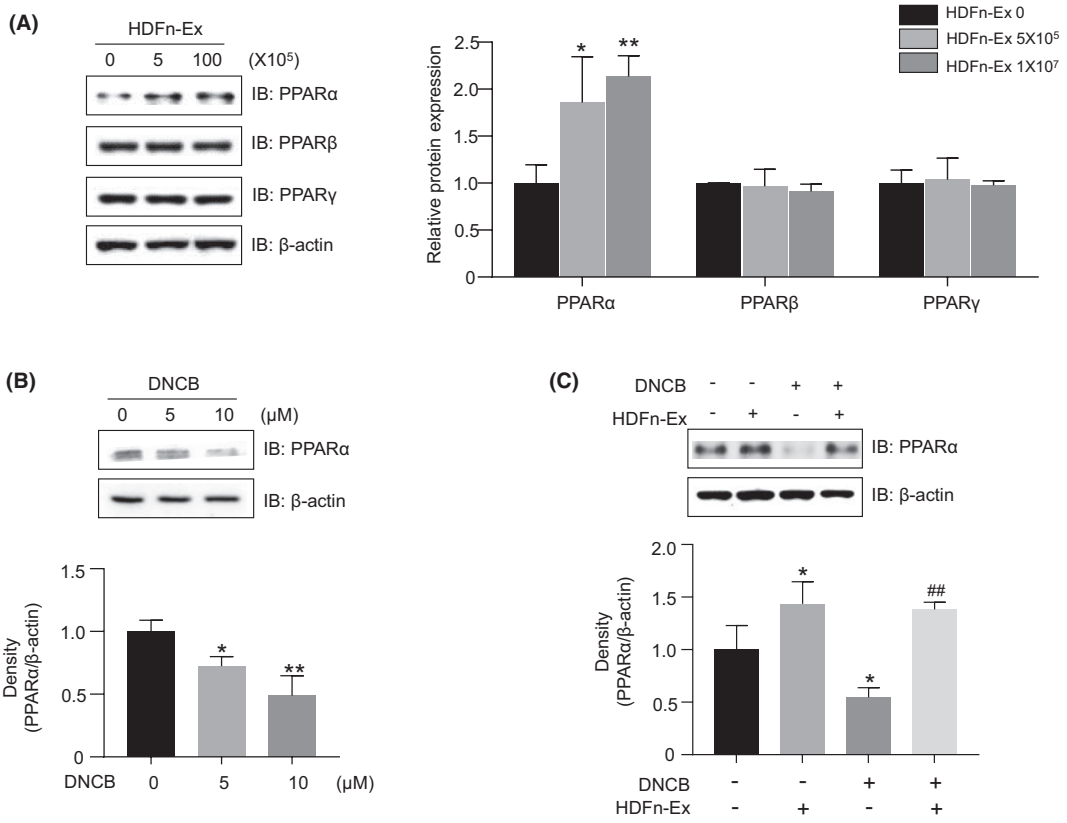

FIGURE 2 Expression of PPARs after HDFn-Ex treatment in HaCaT cells. (A) Expression levels of PPAR isotypes antibodies (PPARα, β, γ) after treatment with HDFn-Ex for 24 h. β-actin was used as an internal control. (B) The expression of PPARα after treatment with HDFn-Ex for 24 h. β-Actin was used as an internal control (C) The expression of PPARα after co-treatment with HDFn-Ex and DNCB for 24 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the controls. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with the DNCB-only group. Unpaired *t*-test, one-way analysis of variance, and post hoc Tukey's test were performed for statistical analyses.

(Normal), while TEWL in Group 3 (DNCB + Dexamethasone) and Group 4 (DNCB + HDFn-Ex) was significantly reduced compared with the Group 2 (DNCB only). In addition, the hydration of SC was lower in the Group 2 (DNCB only) compared with the Group 4 (DNCB + HDFn-Ex). GW6471 abrogated the ability of HDFn-Ex to control skin moisture (Figure 4I,J). Moreover, HDFn-Ex inhibited DNCB-mediated decreases in PPARα, filaggrin and HAS1 levels, and the protective effect of HDFn-Ex on skin barrier was inhibited by GW6471 (Figure 4D–F). Overall, these findings suggest that HDFn-Ex might constitute a potential medicine to treat AD-mediated skin dysfunction.

3.5 | HDFn-Ex exhibits anti-inflammatory properties

To investigate the anti-inflammatory effect of HDFn-Ex in DNCB-induced AD-like mouse model, we assessed the abundance of mast cells using TB staining and the expression of TNF-α. The Group 2 (DNCB only) exhibited increased mast cell infiltration compared to the Group 1 (Normal) (Figure 5A,C). The increase in TNF-α levels induced by DNCB was reversed by HDFn-Ex; however, pre-treatment with GW6471 abrogated this anti-inflammatory effect of HDFn-Ex (Figure 5B). IgE is an important therapeutic target in AD and activates mast cells to release histamine.[29,30] IL-4 also plays a key role in IgE-dependent inflammatory responses.[31] Thus, we analysed IgE and IL-4 levels in serum using ELISA. The levels of IgE and IL-4 were significantly lower in the Group 4 (DNCB + HDFn-Ex). However, the protective effect of HDFn-Ex was not observed in the Group 5 (DNCB + HDFn-Ex + GW6471) (Figure 5D,E). TNF-α induces the transcription of genes regulating inflammation, primarily through activation of the NF-κB pathway.[32] HDFn-Ex inhibited DNCB-mediated increase of TNF-α expression and the phosphorylation of IκBα ($Ser^{32}/^{36}$). In contrast, GW6471 blocked the suppressive effect of HDFn-Ex on DNCB-induced inflammation (Figure 5F,G). These results indicate that HDFn-Ex exhibits anti-inflammatory activity and acts similarly to PPARα activators.

4 | DISCUSSION

Eupatilin, an activator of PPARα, inhibits the development of oxazolone-induced AD symptoms in BALB/c mice.[33] Consistent with the above study, we found that HDFn-Ex has anti-inflammatory activity by regulating PPARα protein levels.

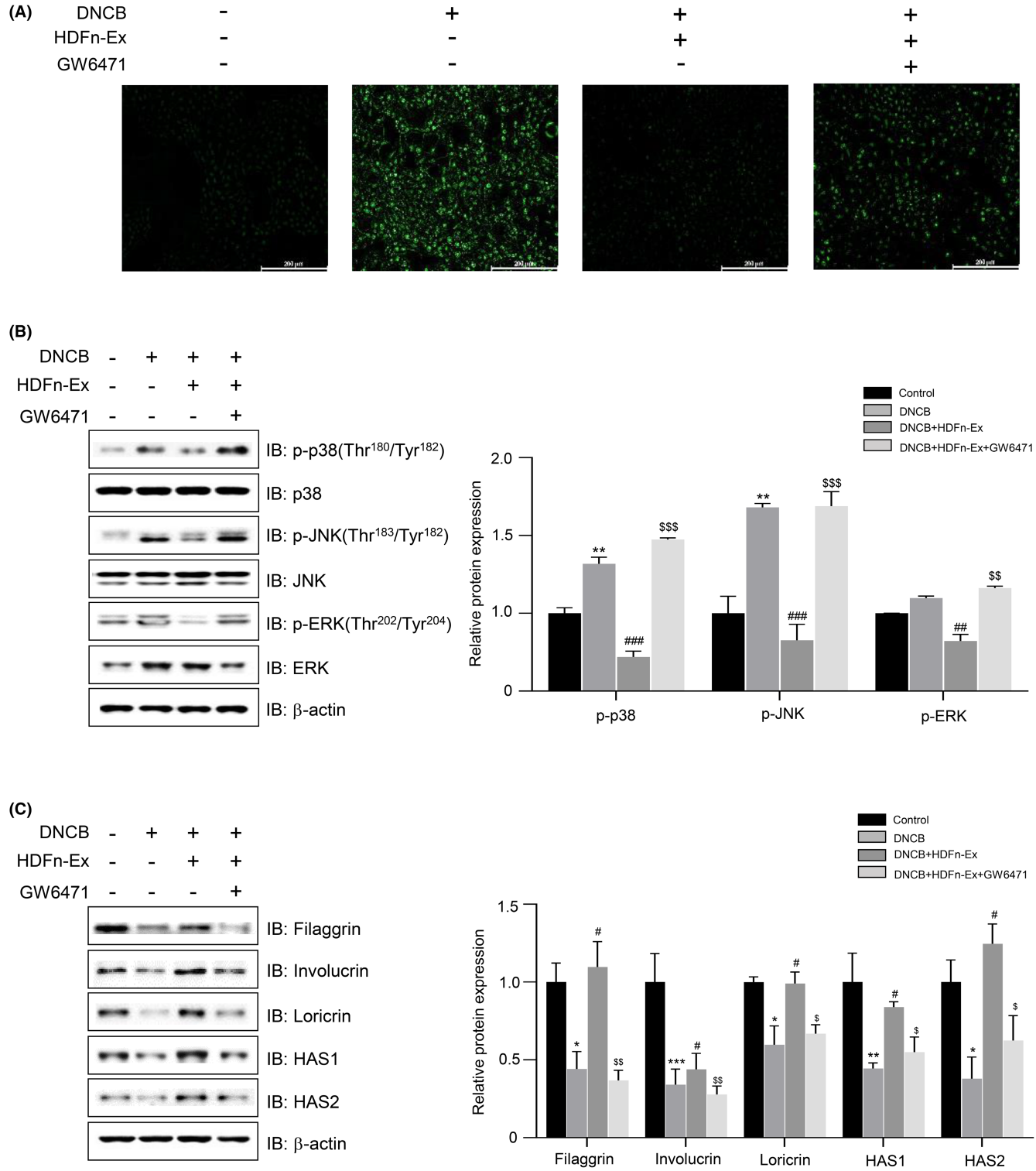

FIGURE 3 HDFn-Ex-mediated regulation of skin barrier after treatment with DNCB. (A) Images (ROS) of cells treated with DNCB (5 μM), HDFn-Ex (1×10^7 particles/mL), and GW6471 (20 μM) for 2 h. Scale bars, 200 μm. (B) The expression of p-p38, p-JNK, and p-ER; p38, JNK, and ERK after co-treatment with DNCB (5 μM), HDFn-Ex (1×10^7 particles/mL), and GW6471 (20 μM) for 3 h. β-Actin was used as an internal control. (C) The expression of filaggrin, involucrin, loricrin, HAS1, and HAS2 after co-treatment with DNCB (5 μM), HDFn-Ex (1×10^7 particles/mL), and GW6471 (20 μM) for 24 h. (D) H&E and IHC staining for (E) PPARα, (F) filaggrin and (G) HAS1 expression in the skin equivalent in vitro models. (H) Epidermal thickness, (I) PPARα, (J) filaggrin and (K) HAS1 density was analysed using the Image J program. Images were captured at 20× magnification. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ compared with the controls. #, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$ compared with the DNCB-only group. $, $p<0.05$; $$, $p<0.01$; $$$, $p<0.001$ compared with the HDFn-Ex group. Unpaired *t*-test, one-way analysis of variance, and *post-hoc* Tukey's test were performed for statistical analyses.

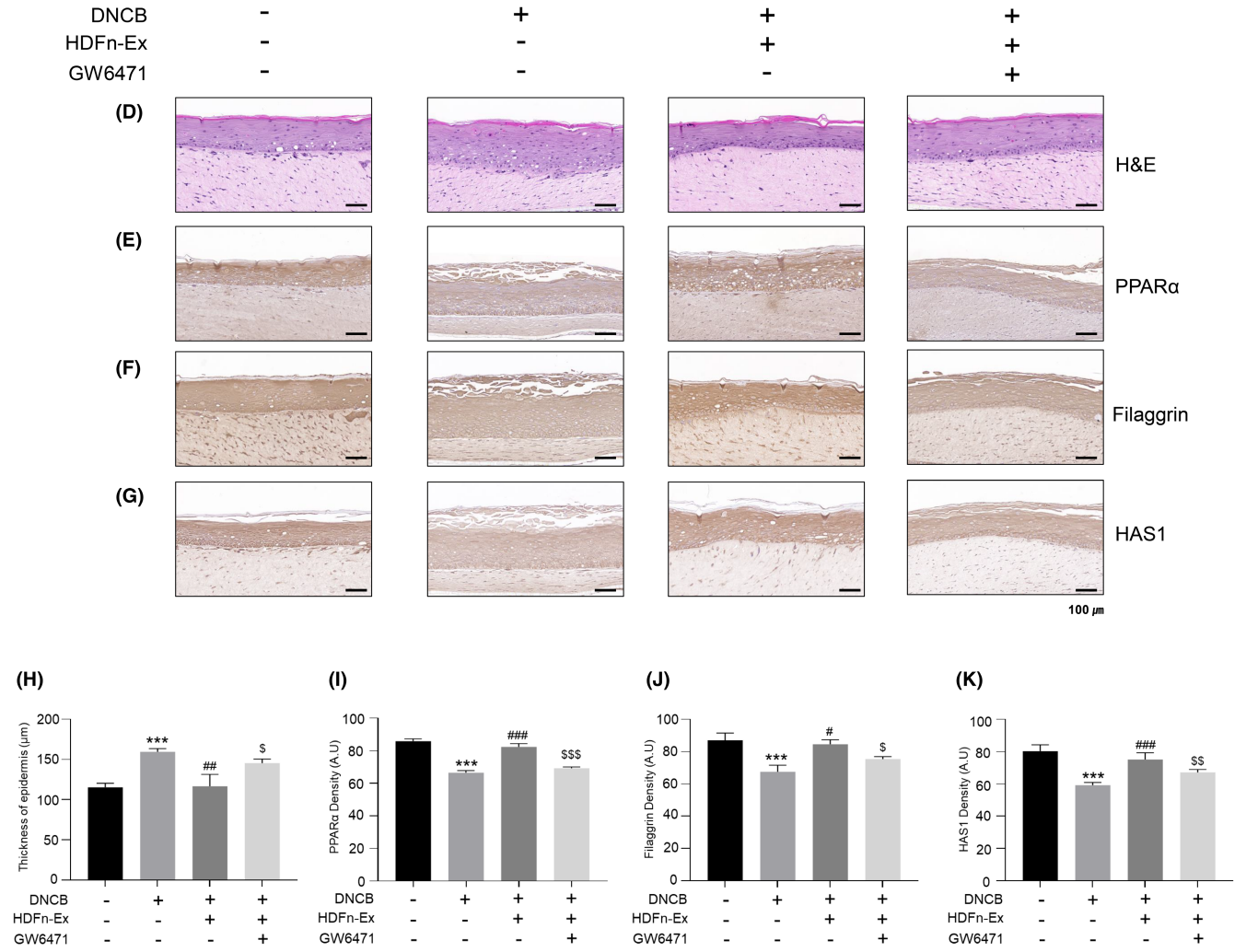

FIGURE 3 (Continued)

The MAPK signalling pathway plays a critical role in epidermal differentiation and skin barrier function.[34] ERK1/2 knockout decreases the expression of terminal keratinocyte differentiation genes.[34] The knockdown of filaggrin influences the MAPK signalling pathway in normal human epidermal keratinocytes.[35] In this study, DNCB leads to a decrease in filaggrin levels and an activation the MAPK signalling pathway (Figure 3B,C). These effects were reversed by HDFn-Ex treatment. These results indicate that HDFn-Ex protects the skin barrier through the filaggrin-MAPK signalling pathway.

Filaggrin, involucrin and loricrin promote the differentiation of keratinocytes and play a key role in the formation and maintenance of the skin barrier function.[36] AD or psoriasis can occur due to the deterioration of the skin barrier function through the downregulation of these proteins.[37] In addition, Th2 cytokines such as interleukin-4, 5 and 13 activate B cells to secrete IgE or prostaglandin D2 (PGD2), and also suppress the expression of loricrin, involucrin and filaggrin.[38] As shown in Figure 3C, HDFn-Ex recovered the expression levels of loricrin, involucrin and filaggrin, which were decreased by DNCB. Notably, these effects were blocked by the PPARα antagonist GW6471. These results indicated that the function of HDFn-Ex is regulated by PPARα.

Retention of HA content is proposed to have an important role in preventing skin aging and improving disease conditions.[39] We found that HDFn-Ex significantly increased HAS1 and HAS2 levels, which were decreased by DNCB; GW6471 treatment reversed the effect of HDFn-Ex (Figure 3C). These observations suggest that HDFn-Ex can improve and strengthen the skin barrier by regulating PPARα expression.

HDFn-Ex improved the dermatitis score, hyperkeratosis and epidermal thickness in the skin of DNCB-induced AD-like mice (Figure 4C,H) and also restored skin barrier function by decreasing TEWL and increasing hydration (Figure 4I,J). In addition, HDFn-Ex increased the expression of PPARα, filaggrin and HAS1 compared to the vehicle group (Figure 4E,F). However, GW6471 abrogated the protective effect of HDFn-Ex on skin barrier. These results imply that HDFn-Ex repairs AD-mediated skin barrier damage via PPARα. HDFn-Ex treatment reduced mast cell numbers, serum IgE and IL4 levels, and TNFα expression in DNCB-induced AD-like mice (Figure 5A–E). This anti-inflammatory activity was suppressed by GW6471.

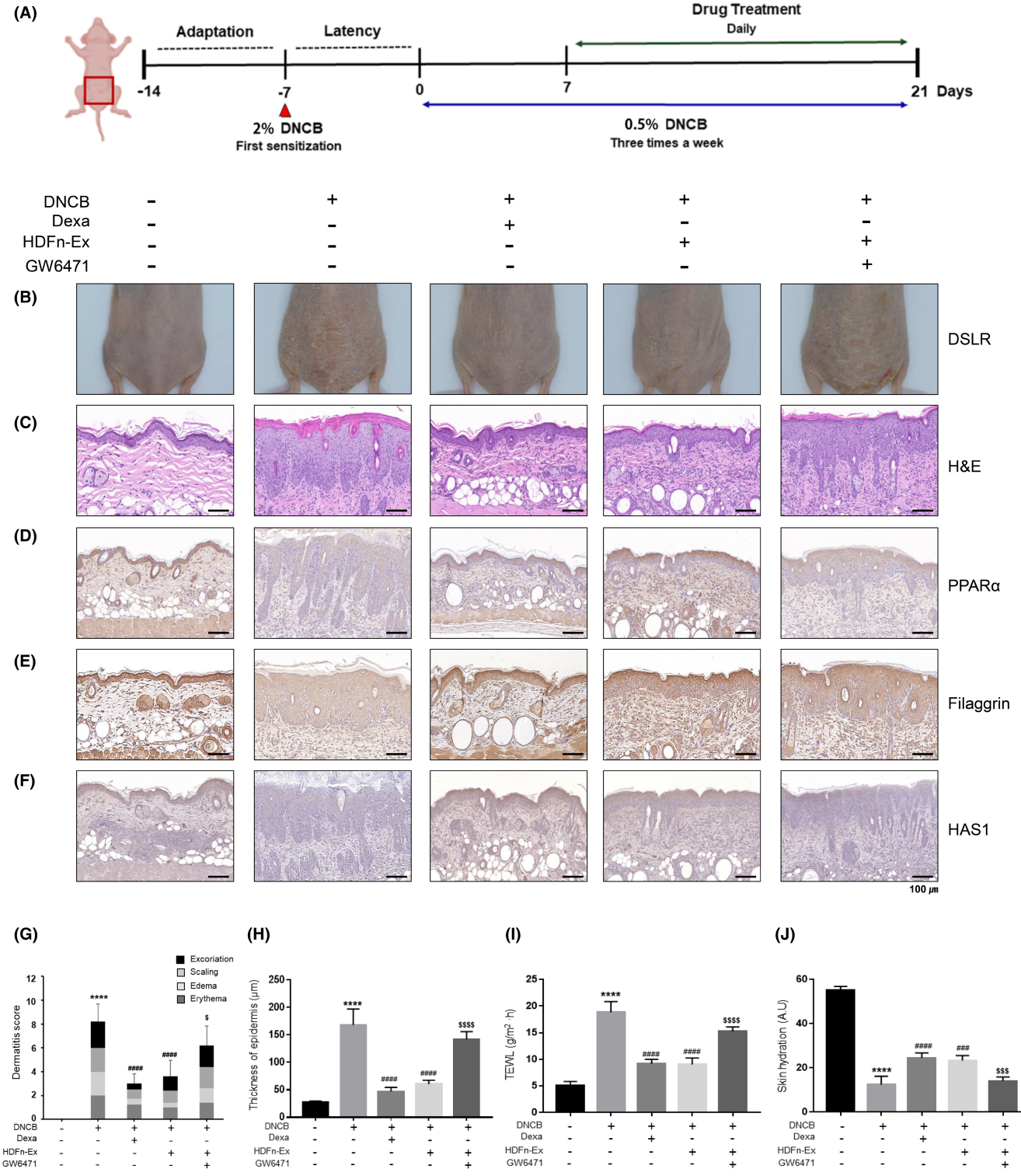

FIGURE 4 HDFn-Ex improves AD induced by repeated exposure to DNCB. (A) Design of the entire experiment. (B) Photography of mouse dorsal skin and (C) H&E staining (20× magnification; Scale bar, 100 μm). (D–F) DAB staining for the expression of PPARα, filaggrin and HAS1. (G) The modified SCORAD score was analysed by combining the signs observed in clinical trials, such as excoriation, scaling, oedema, and erythema, with scores of 0 (clear), 1 (almost clear), 2 (mild), 3 (moderate) and 4 (severe) for each sign. Histological examination of dorsal skin lesion at the end of the experiment. (H) Epidermal thickness, (I) TEWL and (J) hydration levels. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with the controls. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; ####, $p < 0.0001$ compared with the DNCB-only group. $, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$; $$$$, $p < 0.0001$ compared with the HDFn-Ex group. One-way analysis of variance and *post-hoc* Tukey's test were performed for statistical analyses.

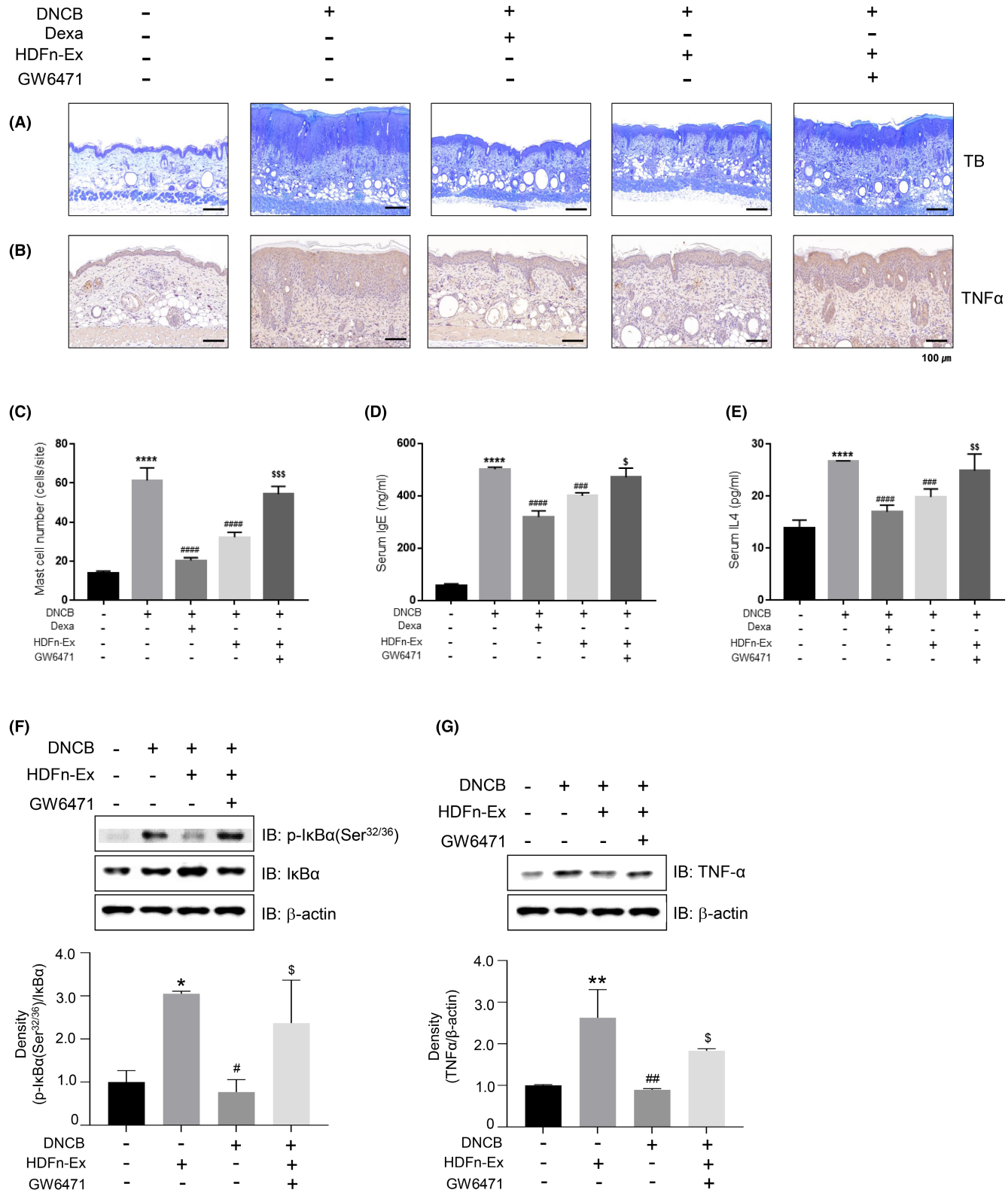

FIGURE 5 HDFn-Ex exhibits anti-inflammatory effects. (A and C) The number of mast cells with dorsal skin lesion was measured using TB staining and quantified using Image J (B) TNF-α expression using DAB staining. (D) ELISA for total serum IgE and (E) IL4 levels. (F and G) The expression of p-IκB and TNF-α with IκBα and β-actin as the control respectively after treatment with co-treated with DNCB (5 μM), HDFn-Ex (1×10^7 particles/mL), and GW6471 (20 μM) for 24 h. (20× magnification; Scale bar, 100 μm *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ compared with the controls. #, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$; ####, $p<0.0001$ compared with the DNCB-only group. $, $p<0.05$; $$, $p<0.01$; $$$, $p<0.001$ compared with the HDFn-Ex group. One-way analysis of variance and post hoc Tukey's test were performed for statistical analyses.

WY-14643, a PPARα agonist, decreases NF-κB pathway activity by modulating the expression, stability, and activity of IκBα.[40] In most unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB proteins that mask the nuclear localization sequence of NF-κB. In response to TNF-α, the IκB kinase (IKK) signalling cascade is activated, leading to the phosphorylation of the N-terminal serine residues Ser^{30} and Ser^{34} of IκBα. This phosphorylation promotes ubiquitination of the IκB proteins, which are subsequently degraded. The degradation of IκB then promotes nuclear translocation of NF-κB.[41] NF-κB activation induces the expression of TNF-α and IL-1β, which exacerbate the acne inflammation.[19] HDFn-Ex inhibited DNCB-induced phosphorylation of IκBα and also HDFn-Ex suppressed the DNCB-induced increase in TNF-α expression (Figure 5F,G). These effects were reversed by GW6471. Taken together, these results demonstrate that HDFn-Ex can inhibit inflammation via the NF-κB/TNF-α signalling pathway. Collectively, we demonstrated for the first time that exosomes from HDFn have the activity to repair AD by upregulating PPARα expression. Therefore, we suggest that HDFn-Ex is an excellent adjuvant with anti-inflammatory actions that restores skin barrier function and improves AD skin symptoms.

5 | CONCLUSIONS

HDFn-Ex alleviated the DNCB-mediated downregulation of filaggrin, involucrin, loricrin, HAS1, and HAS2 in HaCaT cells. However, these effects were inhibited by the PPARα antagonist GW6471. In artificial skin, HDFn-Ex significantly inhibited DNCB-induced epidermal hyperplasia and the decreases in PPARα, filaggrin and HAS1 in a PPARα dependent manner. HDFn-Ex administration reduced thickening of the epidermis and infiltration of mast cells into the dermis compared to DNCB treatment. Moreover, the decreases in PPARα, filaggrin and HAS1 expression levels and increases in IgE and IL4 levels due to DNCB treatment were reversed by HDFn-Ex. These effects of HDFn-Ex were blocked by pre-treatment with GW6471. Furthermore, HDFn-Ex exhibited an anti-inflammatory effect by inhibiting the increases in IκBα phosphorylation and TNF-α expression induced by DNCB. Taken together, our findings indicate that HDFn-Ex exhibits potential for treating AD by recovering damaged skin barrier and activating anti-inflammatory functions.

AUTHOR CONTRIBUTIONS

You Na Jang, Kwang Ho Yoo, Jung Ok Lee, and Beom Joon Kim designed the research. You Na Jang performed the research and acquired the data. Jung Min Lee and A Yeon Park performed the animal experiments and helped analyse the data. You Na Jang and Jung Ok Lee wrote the manuscript and researched the data. You Na Jang and S.Y.K researched the data and contributed to the discussion. Kwang Ho Yoo and Beom Joon Kim contributed to the discussion and reviewed/edited the manuscript. All authors contributed to and approved the final manuscript.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2021R1G1A1007827). This research was also supported by the Chung-Ang University Research Scholarship Grants in 2023.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

You Na Jang https://orcid.org/0000-0002-0014-9783
Jung Ok Lee https://orcid.org/0000-0002-0048-5322
Jung Min Lee https://orcid.org/0000-0002-4670-2521
A. Yeon Park https://orcid.org/0000-0002-4667-8875
Yu Jin Kim https://orcid.org/0000-0002-1247-2887
Su Young Kim https://orcid.org/0000-0002-3251-5813
Joon Seok https://orcid.org/0000-0002-8016-8385
Kwang Ho Yoo https://orcid.org/0000-0002-0137-6849
Beom Joon Kim https://orcid.org/0000-0003-2320-7621

REFERENCES

1. Peng W, Novak N. Pathogenesis of atopic dermatitis. *Clin Exp Allergy*. 2015;45(3):566-574. doi:10.1111/cea.12495
2. Otsuka A, Nomura T, Rerknimitr P, Seidel JA, Honda T, Kabashima K. The interplay between genetic and environmental factors in the pathogenesis of atopic dermatitis. *Immunol Rev*. 2017;278(1):246-262. doi:10.1111/imr.12545
3. Proksch E, Folster-Holst R, Brautigam M, Sepehrmanesh M, Pfeiffer S, Jensen JM. Role of the epidermal barrier in atopic dermatitis. *J Dtsch Dermatol Ges*. 2009;7(10):899-910. doi:10.1111/j.1610-0387.2009.07157.x
4. Nicol NH. Anatomy and physiology of the skin. *Dermatol Nurs*. 2005;17(1):62.
5. Baroni A, Buommino E, De Gregorio V, Ruocco E, Ruocco V, Wolf R. Structure and function of the epidermis related to barrier properties. *Clin Dermatol*. 2012;30(3):257-262. doi:10.1016/j.clindermatol.2011.08.007
6. Christofides A, Konstantinidou E, Jani C, Boussiotis VA. The role of peroxisome proliferator-activated receptors (PPAR) in immune responses. *Metabolism*. 2021;114:154338. doi:10.1016/j.metabol.2020.154338
7. Schmuth M, Moosbrugger-Martinz V, Blunder S, Dubrac S. Role of PPAR, LXR, and PXR in epidermal homeostasis and inflammation. *Biochim Biophys Acta*. 2014;1841(3):463-473. doi:10.1016/j.bbalip.2013.11.012
8. Chon SH, Tannahill R, Yao X, Southall MD, Pappas A. Keratinocyte differentiation and upregulation of ceramide synthesis induced by an oat lipid extract via the activation of PPAR pathways. *Exp Dermatol*. 2015;24(4):290-295. doi:10.1111/exd.12658
9. Majewski G, Craw J, Falla T. Accelerated barrier repair in human skin explants induced with a plant-derived PPAR-alpha activating complex via cooperative interactions. *Clin Cosmet Investig Dermatol*. 2021;14:1271-1293. doi:10.2147/CCID.S325967
10. Rivier M, Castiel I, Safonova I, Ailhaud G, Michel S. Peroxisome proliferator-activated receptor-alpha enhances lipid metabolism in

a skin equivalent model. *J Invest Dermatol*. 2000;114(4):681-687. doi:10.1046/j.1523-1747.2000.00939.x
11. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255-289. doi:10.1146/annurev-cellbio-101512-122326
12. Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci*. 2000;113(Pt 19):3365-3374. doi:10.1242/jcs.113.19.3365
13. Thery C. Exosomes: secreted vesicles and intercellular communications. *F1000 Biol Rep*. 2011;3:15. doi:10.3410/B3-15
14. Record M, Carayon K, Poirot M, Silvente-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. *Biochim Biophys Acta*. 2014;1841(1):108-120. doi:10.1016/j.bbalip.2013.10.004
15. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated t ransfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9(6):654-659. doi:10.1038/ncb1596
16. Zhao L, Liu W, Xiao J, Cao B. The role of exosomes and "exosomal shuttle microRNA" in tumorigenesis and drug resistance. *Cancer Lett*. 2015;356(2 Pt B):339-346. doi:10.1016/j.canlet.2014.10.027
17. Yang D, Zhang W, Zhang H, et al. Progress, opportunity, and perspective on exosome isolation–efforts for efficient exosome-based theranostics. *Theranostics*. 2020;10(8):3684-3707. doi:10.7150/thno.41580
18. Han X, Wu P, Li L, et al. Exosomes derived from autologous dermal fibroblasts promote diabetic cutaneous wound healing through the Akt/beta-catenin pathway. *Cell Cycle*. 2021;20(5–6):616-629. doi:10.1080/15384101.2021.1894813
19. Zhou BR, Zhang JA, Zhang Q, et al. Palmitic acid induces production of proinflammatory cytokines interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha via a NF-kappaB-dependent mechanism in HaCaT keratinocytes. *Mediators Inflamm*. 2013;2013:530429. doi:10.1155/2013/530429
20. Cho BS, Kim JO, Ha DH, Yi YW. Exosomes derived from human adipose tissue-derived mesenchymal stem cells alleviate atopic dermatitis. *Stem Cell Res Ther*. 2018;9(1):187. doi:10.1186/s13287-018-0939-5
21. Ghosalkar S, Singh P, Ravikumar P. Emerging topical drug delivery approaches for the treatment of atopic dermatitis. *J Cosmet Dermatol*. 2022;21(2):536-549. doi:10.1111/jocd.14685
22. Queille C, Pommarede R, Saurat JH. Efficacy versus systemic effects of six topical steroids in the treatment of atopic dermatitis of childhood. *Pediatr Dermatol*. 1984;1(3):246-253. doi:10.1111/j.1525-1470.1984.tb01124.x
23. Xie F, Zhou X, Fang M, et al. Extracellular vesicles in cancer immune microenvironment and cancer immunotherapy. *Adv Sci (Weinh)*. 2019;6(24):1901779. doi:10.1002/advs.201901779
24. Kang H, Lee CH, Kim JR, et al. Chlorella vulgaris attenuates Dermatophagoides Farinae-induced atopic dermatitis-like symptoms in NC/Nga mice. *Int J Mol Sci*. 2015;16(9):21021-21034. doi:10.3390/ijms160921021
25. Yamashina M, Takami T, Kanemura T, Orii T, Ojima A. Immunohistochemical demonstration of complement components in formalin-fixed and paraffin-embedded renal tissues. *Lab Invest*. 1989;60(2):311-316.
26. Neo JRE, Teo ZN, Yeo JSE, et al. Tocotrienols improve urban particulate matter-induced skin damages by regulating skin barrier function and ROS/MAPK signalling pathway in keratinocytes. *Atmos Pollut Res*. 2022;13(10):101564. doi:10.1016/j.apr.2022.101564
27. Papakonstantinou E, Roth M, Karakiulakis G. Hyaluronic acid: a key molecule in skin aging. *Dermatoendocrinol*. 2012;4(3):253-258. doi:10.4161/derm.21923
28. Lee JH, Chen SY, Yu CH, et al. Noninvasive in vitro and in vivo assessment of epidermal hyperkeratosis and dermal fibrosis in atopic dermatitis. *J Biomed Opt*. 2009;14(1):014008. doi:10.1117/1.3077182
29. Liu FT, Goodarzi H, Chen HY. IgE, mast cells, and eosinophils in atopic dermatitis. *Clin Rev Allergy Immunol*. 2011;41(3):298-310. doi:10.1007/s12016-011-8252-4
30. Leung DY. Role of IgE in atopic dermatitis. *Curr Opin Immunol*. 1993;5(6):956-962. doi:10.1016/0952-7915(93)90112-6
31. Izuhara K, Shirakawa T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int J Mol Med*. 1999;3(1):3-10. doi:10.3892/ijmm.3.1.3
32. Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. *Nat Rev Immunol*. 2008;8(11):837-848. doi:10.1038/nri2423
33. Jung Y, Kim JC, Park NJ, et al. Eupatilin, an activator of PPARalpha, inhibits the development of oxazolone-induced atopic dermatitis symptoms in Balb/c mice. *Biochem Biophys Res Commun*. 2018;496(2):508-514. doi:10.1016/j.bbrc.2018.01.098
34. Meng X, Qiu L, Song H, Dang N. MAPK pathway involved in epidermal terminal differentiation of Normal human epidermal keratinocytes. *Open Med (Wars)*. 2018;13:189-195. doi:10.1515/med-2018-0029
35. Wang S, Qiu L, Meng X, Dang N. Knock-down of filaggrin influences the mitogen-activated protein kinases signaling pathway in normal human epidermal keratinocytes. *Med Sci (Paris)*. 2018;34 Focus issue(F1):94-98. doi:10.1051/medsci/201834f116
36. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. *Exp Dermatol*. 2008;17(12):1063-1072. doi:10.1111/j.1600-0625.2008.00786.x
37. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis–part I: clinical and pathologic concepts. *J Allergy Clin Immunol*. 2011;127(5):1110-1118. doi:10.1016/j.jaci.2011.01.053
38. Furue M. Regulation of Filaggrin, Loricrin, and Involucrin by IL-4, IL-13, IL-17A, IL-22, AHR, and NRF2: pathogenic implications in atopic dermatitis. *Int J Mol Sci*. 2020;21(15), 5382. doi:10.3390/ijms21155382
39. Kavasi RM, Berdiaki A, Spyridaki I, et al. HA metabolism in skin homeostasis and inflammatory disease. *Food Chem Toxicol*. 2017;101:128-138. doi:10.1016/j.fct.2017.01.012
40. Briguglio E, Di Paola R, Paterniti I, et al. WY-14643, a potent peroxisome proliferator activator receptor-alpha PPAR-alpha agonist ameliorates the inflammatory process associated to experimental periodontitis. *PPAR Res*. 2010;2010:193019. doi:10.1155/2010/193019
41. Bougarne N, Weyers B, Desmet SJ, et al. Molecular actions of PPAR alpha in lipid metabolism and inflammation. *Endocr Rev*. 2018;39(5):760-802. doi:10.1210/er.2018-00064

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Effect of PPARα on HDFn-Ex mediated skin barrier related protein levels after treatment with DNCB. Western blot data show that PPARα siRNA inhibited on HDFn-Ex inducing skin barrier markers changes. HaCaT cells were transfected with PPARα siRNA (Dharmacon, ON-TARGET plus siRNA SMART Pool) 24 h prior

to incubation with DNCB (5 μM), HDFn-Ex (1 × 107 particles/mL) for 24 h. HDFn-Ex significantly increased filaggrin, involucrin, loricrin, HAS1 and HAS2. PPARα siRNA but not non-target siRNA (Bioneer, Daejeon, Korea) inhibited the HDFn-Ex induced changes. The data are expressed *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ compared with the controls. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with the DNCB-only group. $, $p < 0.05$; $$, p < 0.01; $$$, $p < 0.001$ compared with the DNCB+HDFn-Ex group. One-way analysis of variance, and post hoc Tukey's test were performed for statistical analyses.

How to cite this article: Jang YN, Lee JO, Lee JM, et al. Exosomes derived from human dermal fibroblasts (HDFn-Ex) alleviate DNCB-induced atopic dermatitis (AD) via PPARα. *Exp Dermatol*. 2024;33:e14970. doi:10.1111/exd.14970