



## ORIGINAL ARTICLE

# The Effect of Adiponectin on the Regulation of Filaggrin Expression in Normal Human Epidermal Keratinocytes

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**Background:** Adiponectin, an adipokine secreted from adipocytes, affects energy metabolism and also shows anti-diabetic and anti-inflammatory properties. Recent studies have reported that adiponectin plays a role in regulating skin inflammation. **Objective:** This study aimed to investigate the effect of adiponectin on the expression of *filaggrin* (FLG) in normal human epidermal keratinocytes (NHEKs). **Methods:** NHEKs were serum-starved for 6h before being treated with adiponectin. Afterward, cell viability was assessed by MTT assay. We also treated with calcium, interleukin (IL)-4, and IL-13 to provide positive and negative comparative controls, respectively. Gene mRNA expression was quantified using real time reverse transcription polymerase chain reaction, and protein expression was evaluated using Western blot. To evaluate the relationship among mitogen-activated protein kinases (MAPKs), activator protein 1 (AP-1), and FLG, we also treated cells with inhibitors for MAPKs JNK, p38, and ERK1/2. **Results:** FLG and FLG-2 mRNA expression in NHEKs significantly increased after treatment with 10  $\mu$ g/ml adipo-

nectin. Adiponectin also restored FLG and FLG-2 mRNA expression that was otherwise inhibited by treatment with IL-4 and IL-13. Adiponectin induced FLG expression via AP-1 and MAPK signaling. **Conclusion:** Adiponectin positively regulated the expression of FLG and could be useful as a therapeutic agent to control diseases related to disrupted skin barrier function. (*Ann Dermatol* 30(6) 645 ~ 652, 2018)

**-Keywords-**

Adiponectin, Cell differentiation, Filaggrin, Keratinocytes, Mitogen-activated protein kinases, Transcription factor AP-1

## INTRODUCTION

Adiponectin is an adipokine protein that is primarily secreted from adipocytes<sup>1</sup>. It is closely related to energy metabolism process and involved in lipid regulation, insulin sensitivity, and cardiovascular functions<sup>2-4</sup>. Additionally, adiponectin possesses anti-inflammatory and anti-atherogenic properties<sup>5,6</sup>.

There have been several recent studies characterizing the effect of adiponectin treatment on skin. In addition to modulating the proliferation, migration, and cytokine secretion of keratinocytes, adiponectin regulates the cutaneous wound healing process<sup>7-9</sup> and the growth and differentiation of keratinocytes<sup>10</sup>. Furthermore, adiponectin plays a role in regulating skin inflammation such as psoriasis<sup>11,12</sup>. Beyond the anti-inflammatory effect of adiponectin on the skin, it may also provide protection against ultraviolet-induced dermal matrix degradation in photoaged human skin<sup>13,14</sup>.

The *filaggrin* (FLG) protein is known to play a key role in maintaining skin barrier function<sup>15</sup>. The levels of FLG pro-

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tein and the products of its breakdown are important for skin barrier function, and common loss-of-function mutations in the *FLG* gene are the strongest known risk factor for atopic dermatitis (AD)<sup>16,17</sup>.

We previously reported that adiponectin upregulates *FLG* expression through an SIRT1 (silent mating type information regulation 2 homolog 1)-mediated pathway and suggested that adiponectin might be a promising agent for improving skin barrier function<sup>18</sup>. This study aimed to explore an additional mechanism by which adiponectin promotes expression of *FLG* in normal human epidermal keratinocytes (NHEKs).

## MATERIALS AND METHODS

### General lab chemicals and preparation

NHEKs, cell culture media (EpiLife, with calcium), human keratinocyte growth serum, and other cell culture materials were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA). Recombinant human interleukin (IL)-4 and IL-13 (variant), produced in *Escherichia coli*, were purchased from Peprotech (Rocky Hill, NJ, USA). Specific antibodies used for Western blot analysis were purchased from Cell Signaling Technology (Danvers, MA, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA). The pharmacologic inhibitors of c-Jun amino-terminal kinase (JNK) (SP600125), p38 (SB203580), and extracellular signal-regulated kinases 1/2 (ERK1/2) (PD98059) were purchased from Calbiochem (San Diego, CA, USA).

### Cell culture and viability assay

NHEKs were maintained in a 5% CO<sub>2</sub> humidified atmosphere at 37°C in EpiLife media supplemented with calcium. The cells were collected at the second or third passage for further experimental use. Prior to all experiments, cells were conditioned for between 4 and 6 h in serum-free media. Cell viability was measured using MTT reduction assays, as described previously<sup>19</sup>. Briefly, cells were incubated for at least 24 h in 96-well plates and pre-treated with different concentrations of adiponectin (2.5, 5, 10, and 20 μg/ml) for an additional 24 h, 48 h, or 72 h. Afterward the culture supernatants were removed, and the resulting purple formazan was dissolved in dimethyl sulfoxide<sup>20</sup>. Absorbance values were measured at 562 nm using a microplate spectrophotometer, SpectraMax340pc (Molecular Devices, Sunnyvale, CA, USA). Cell viability was calculated relative to the absorbance of the adiponectin untreated normal control (NC) group.

### Western blot analysis

Standard procedures were used for all Western blotting analysis. Briefly, cells were lysed in 1% Triton-X radio-immunoprecipitation assay buffer for 1 min. Cellular debris was removed by centrifugation at 13,200 rpm for 15 min. Lysate protein concentration was determined using the bicinchoninic acid assay method. Cell lysates that contained equal amounts of protein (~35 μg of total protein) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electro-blotted onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin and then incubated with the desired primary and secondary antibodies. Protein expression was detected using the EzWestLumi plus system (ATTO, Tokyo, Japan), according to the manufacturer's instructions. Blots were visualized using a ChemiDocTM XRS image analyzer (Bio-Rad, Hercules, CA, USA), and protein expression levels were quantified using ImageJ software and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin.

### RNA extraction and quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis

Total RNA was extracted from keratinocytes using TRIzol<sup>®</sup> reagent (Wegene, Seoul, Korea) following the manufacturer's recommended protocol. The cDNA synthesized from mRNA was then incubated for an additional 1 h at 42°C. Single-stranded cDNA was amplified by PCR with specific primers for genes encoding *FLG*, *FLG-2*, *GAPDH*, and *cyclophilin A* (*CypA*). Initial mRNA quantities were determined after performing real-time PCR using a thermal cycler (Bio-Rad) with SYBR premix Ex Taq (Takara, Shiga, Japan) following the manufacturer's recommended protocol. All measurements include duplicate experiments, and the results were analyzed using the  $2^{-\Delta\Delta CT}$  method. Expression data were normalized to *GAPDH* and *CypA*.

### Positive and negative controls for *FLG* expression

Because *FLG* is involved in the terminal differentiation of keratinocytes to form the cornified cell envelope<sup>21</sup>, a 1.2 mM concentration of calcium (Ca<sup>2+</sup>), as a positive control for *FLG* expression. Previous studies have shown that IL-4 and IL-13 inhibit *FLG* and *FLG-2* gene expression, respectively<sup>22,23</sup>. Recombinant IL-4 (50 ng/ml) and IL-13 (50 ng/ml) were added to the keratinocyte culture media for 5 days in order to provide a negative experimental control for *FLG* expression.

## Statistical analysis

All in vitro data are presented as the mean  $\pm$  standard deviation (SD). The mean values were calculated based on data from at least three independent replicate experiments that were conducted on separate days using freshly prepared reagents. Data were analyzed using paired t-test. Significant differences were defined at  $p$ -value  $< 0.05$ . All statistical analyses were performed using PASW SPSS ver. 18.0 (IBM Co., Armonk, NY, USA).

## RESULTS

### The effect of adiponectin on the differentiation of keratinocytes

Adiponectin induced no cytotoxicity up to a concentration of 10  $\mu$ g/ml. However, cell viability was significantly reduced when NHEKs were treated with a concentration of 20  $\mu$ g/ml adiponectin for 24 h (Fig. 1A). Protein and mRNA expression of *involucrin* and *FLG*, which are common markers of keratinocyte differentiation, was increased by adiponectin in a dose-dependent manner (Fig. 1B). As such, all subsequent experiments were conducted using a concentration of 10  $\mu$ g/ml adiponectin.

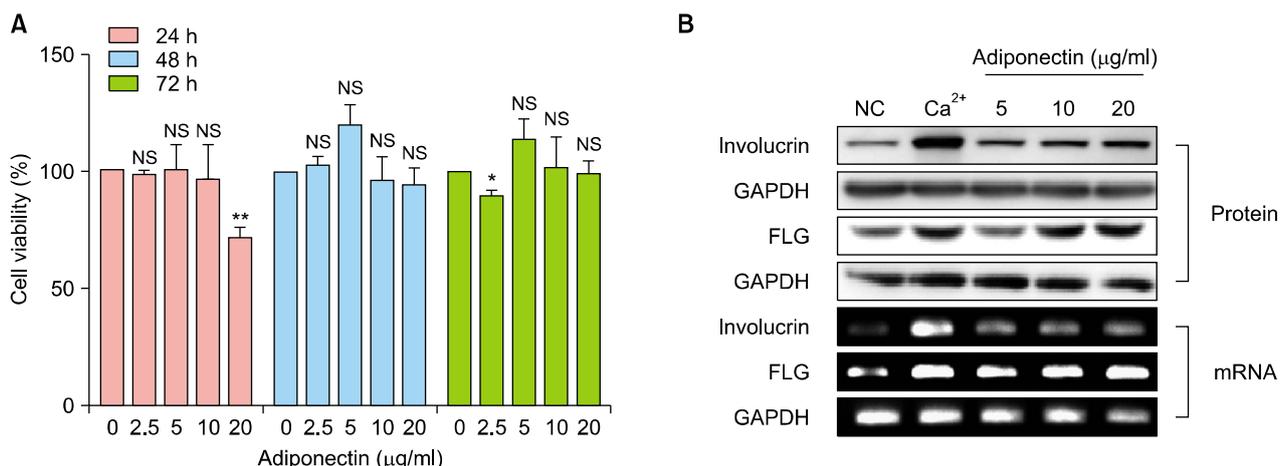
### Adiponectin induces *FLG* mRNA expression

In the analysis of the transcriptional level of *FLG* using RT-PCR following a time course of up to 120 h of adiponectin treatment, the relative mRNA level of *FLG* was sig-

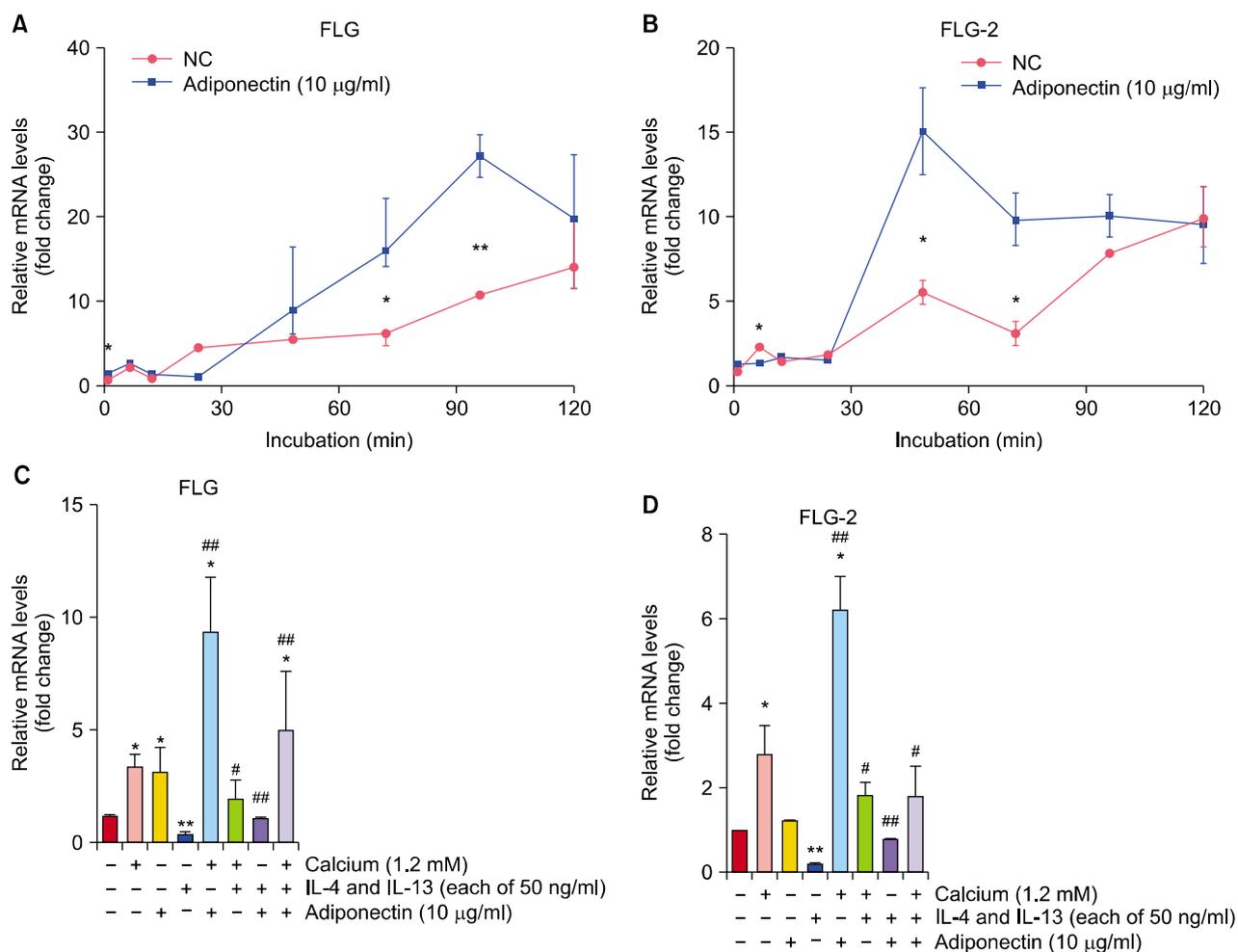
nificantly higher 72 h and 96 h after the start of adiponectin incubation (Fig. 2A). The relative mRNA level of *FLG-2* was significantly higher between 48 h and 72 h after the start of incubation with adiponectin (Fig. 2B). These results suggest that adiponectin simultaneously promotes the transcriptional upregulation of *FLG* and *FLG-2*.

### Adiponectin restores *FLG* mRNA expression reduced by IL-4 and IL-13

Both IL-4 and IL-13 significantly inhibited *FLG* and *FLG-2* gene expression compared with the NC. The simultaneous addition of adiponectin along with IL-4 and IL-13, however, successfully reversed their inhibition of *FLG* and *FLG-2* gene expression (Fig. 2C, D). These results show that adiponectin restores *FLG* and *FLG-2* mRNA expression under normally inhibitory conditions. Interestingly, the simultaneous addition of adiponectin and  $Ca^{2+}$  augmented the inductive action of  $Ca^{2+}$  on *FLG* and *FLG-2* gene expression, demonstrating that adiponectin exerts a synergistic effect with  $Ca^{2+}$  on *FLG* and *FLG-2* mRNA expression. As shown in Fig. 3B, adiponectin and  $Ca^{2+}$  were differently phosphorylation of mitogen-activated protein kinases (MAPKs) protein and the treatment of adiponectin and  $Ca^{2+}$  together was synergistic up-regulated phosphorylation of ERK.



**Fig. 1.** The effect of adiponectin on the differentiation of keratinocytes. (A) Keratinocyte cell viability after adiponectin treatment at 24 h, 48 h, and 72 h. Cells were serum-starved for 6 h before treatment with adiponectin (2.5, 5, 10, and 20  $\mu$ g/ml). Cell viability was assessed by MTT assay and expressed as a percentage of the control (untreated) group. (B) Protein and mRNA expression of keratinocyte differentiation markers. Cells were treated with adiponectin (5, 10, and 20  $\mu$ g/ml) for 72 h. Protein and mRNA expression was analyzed by Western blot and reverse transcription-polymerase chain reaction, respectively. Data are presented as the mean  $\pm$  standard deviation of three independent replicate experiments ( $n=3$ ). NS: no significance, NC: normal control group, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, *FLG*, filaggrin.  $**p < 0.005$ , control vs. adiponectin treatment group.



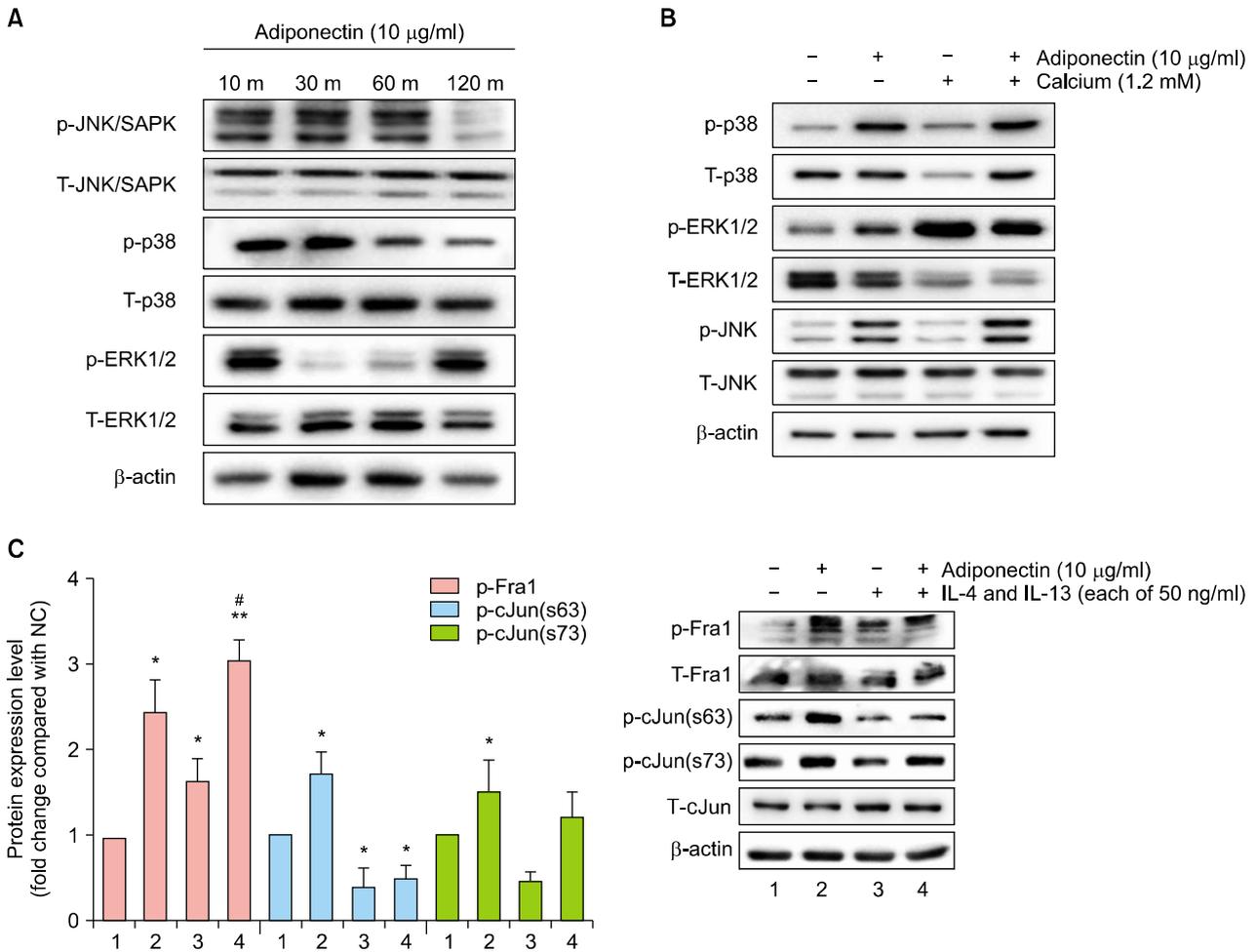
**Fig. 2.** Adiponectin induces *filaggrin* (*FLG*) and *FLG-2* mRNA expression. The time dependent relative mRNA expression after adiponectin treated of (A) *FLG* and (B) *FLG-2* was analyzed by real time reverse transcription-polymerase chain reaction. Data are represented in graphical form and show the fold change compared to normal control (NC) cells of the 1 h incubation group. The protective effect of adiponectin on (C) *FLG* and (D) *FLG-2* mRNA expression under normally inhibitory treatment with Th2 cytokines interleukin (IL)-4 and IL-13. Calcium (1.2 mM) was used as a positive control for *FLG* expression. Data are presented in graphical form and show the fold change compared to NC cells. *CypA* expression levels were used as an internal control. Data are presented as the mean ± standard deviation of three independent replicate experiments (n=3). (A, B) \**p*<0.05, \*\**p*<0.005, NC group vs. adiponectin-treated group. (C, D) \**p*<0.05; \*\**p*<0.005 vs. NC; #*p*<0.05, ##*p*<0.005 vs. IL-4- and IL-13-treated group.

### Adiponectin regulation of *FLG* expression depends on the activator protein 1 (AP-1) components c-Jun, Fra1, and MAPK signaling

After 10 μg/ml adiponectin treatment, c-Jun and Fra1 proteins were significantly phosphorylated in NHEKs. When NHEKs were treated with adiponectin as well as IL-4 and IL-13, c-Jun (serine 73) and Fra-1 were more phosphorylated compared to their levels after treatment with IL-4 and IL-13 alone (Fig. 3C). This finding suggests that adiponectin may act to regulate the expression of c-Jun and Fra1 proteins, which are both components of the AP-1 transcription factor. Expression of c-Fos protein, however, was

not detected (data not shown).

Adiponectin induced the time-dependent phosphorylation of MAPKs (Fig. 3A). When the JNK inhibitor SP600125 (at 10 μM), p38 inhibitor SB203580 (at 10 μM), and ERK inhibitor PD98059 (at 10 μM) were respectively added to NHEKs 1 hour before also adding 10 μg/ml of adiponectin, phosphorylation of JNK/SAPK, p38, and ERK was attenuated (Fig. 4A). The phosphorylation of c-Jun (serine 73) was significantly lower in conditions with the ERK inhibitor, and the phosphorylation of Fra1 was significantly lower in conditions with the JNK and ERK inhibitors (Fig. 4B). The transcriptional levels of *FLG* and *FLG-2* were most effectively attenuated by applying p38 and ERK in-



**Fig. 3.** Adiponectin induced phosphorylation of mitogen-activated protein kinases (MAPKs) and activator protein 1. (A) The time-dependent phosphorylation of MAPKs was induced by adiponectin treatment (10  $\mu$ g/ml). (B) Adiponectin and calcium (1.2 mM) differently phosphorylation of MAPKs proteins. Adiponectin regulated the phosphorylation of (C) Fra-1 and c-Jun protein expression. Protein and mRNA expression levels were analyzed by Western blot and reverse transcription-polymerase chain reaction, respectively. Data are presented in graphical form and show the fold change compared with sub-confluent normal control (NC) cells. Data are presented as the mean  $\pm$  standard deviation of three independent replicate experiments (n = 3). \* $p$  < 0.05, \*\* $p$  < 0.005 vs. NC group; # $p$  < 0.05 vs. IL-4- and IL-13-treated group.

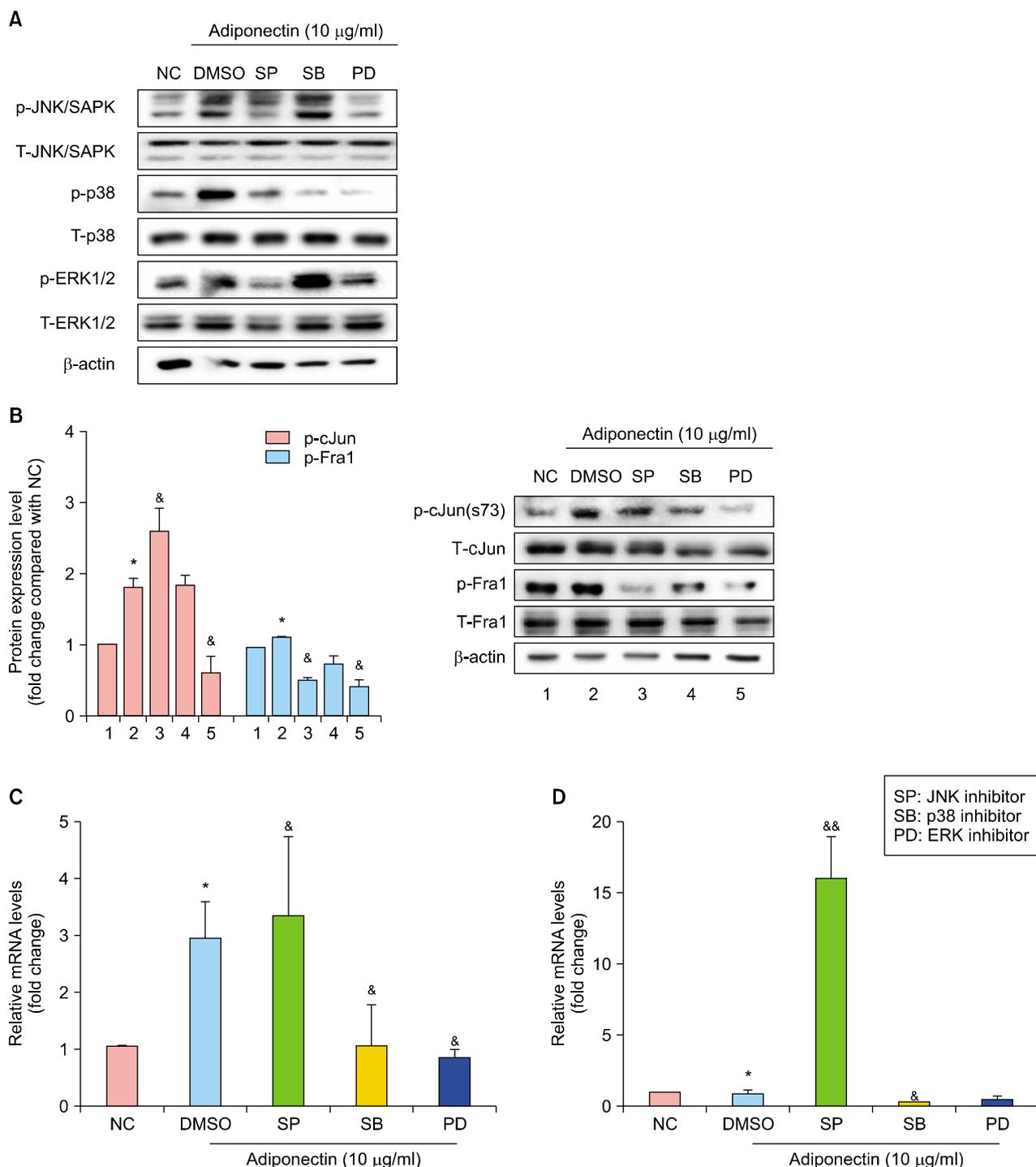
hibitors with adiponectin (Fig. 4C, D).

## DISCUSSION

Subcutaneous tissue, including adipose tissue, serves many important functions and critically regulates skin homeostasis and pathophysiology. Accordingly, adipose tissue is sometimes referred to as a cytokine depot, which can then affect the epidermis and dermis via endocrine, paracrine, and autocrine pathways<sup>24</sup>. Many recent dermatologic studies have suggested that, among the many different adipokines, adiponectin in particular may have beneficial effects on the skin. Normal human keratinocytes express adiponectin receptors AdipoR1 and AdipoR2<sup>8</sup>, and

adiponectin is known to regulate the proliferation and migration of keratinocytes<sup>9</sup>, as well as play a crucial role in the regulation of cutaneous wound healing and skin inflammation<sup>11,12</sup>.

*FLG* and its high molecular-weight precursor profilaggrin (proFLG) are filament-associated proteins that aggregate keratin fibers in keratinocytes. The cellular processing of proFLG and *FLG* provides an important material source of natural moisturizing factors (NMF), and multiple proteolytic enzymes have been implicated in their proteolytic processing<sup>25</sup>. *FLG-2* shares numerous properties with *FLG*, including a closely related structural organization, an identical pattern of expression and localization in the epidermis, and analogous proteolysis processing. As such, it



**Fig. 4.** Adiponectin regulation of filaggrin (*FLG*) mRNA expression depends on the activator protein 1 components c-Jun and fos related antigen-1 (Fra-1) and mitogen-activated protein kinase (MAPK) signaling. (A) MAPK inhibitors attenuated the phosphorylation of JNK/SAPK, ERK and p38. The relative (B) protein expression of Fra-1 and cJun and mRNA expression of (C) *FLG* and (D) *FLG-2* after treatment with adiponectin and MAPK inhibitors. Normal human epidermal keratinocytes were treated with MAPK inhibitors SP600125, SB203580, and PD98059 before adiponectin treatment. Protein and mRNA expression levels were analyzed by Western blot and reverse transcription-polymerase chain reaction, respectively. Data are presented in graphical form and show the fold change compared with sub-confluent normal control (NC) cells. Data are presented as the mean ± standard deviation of three independent replicate experiments (n=3). DMSO: used as vehicle, dimethyl sulfoxide, SP: SP600125, JNK inhibitor, SB: SB203580, p38 inhibitor, PD: PD98059, ERK inhibitor. \* $p < 0.05$ , \*\* $p < 0.005$  vs. NC group; & $p < 0.05$ , && $p < 0.005$  vs. adiponectin-treated group.

is understood that *FLG-2* and *FLG* have overlapping and synergistic roles driving the formation of the epidermal barrier by degradation to NMF<sup>26,27</sup>.

Our recent study showed that adiponectin upregulates *FLG* expression through a SIRT1-mediated pathway, suggesting that adiponectin might be a promising agent for enhancing skin barrier function<sup>18</sup>. We demonstrated that adiponectin downregulates *hBD2* mRNA expression through the suppression of p38 and JNK/SAPK MAPK signaling in a previous study<sup>28</sup> and performed the present study based on the assumption that adiponectin would have additional signaling mechanisms that promote the expression of *FLG*.

We first quantified changes in mRNA expression of *FLG* in NHEKs treated with adiponectin. We then demonstrated that adiponectin exerts a protective effect on the increased level of *FLG* expression by reversing the inhibition of expression due to treatment with Th2 cytokines IL-4 and IL-13. Our data indicate that adiponectin not only has the ability to promote *FLG* mRNA expression, but can also prevent inhibition of *FLG* expression in the Th2 cytokine milieu, such as AD.

AP-1 plays several key roles in the regulation of cell proliferation and differentiation as a regulatory transcription factor<sup>29</sup>. AP-1 functions as a homodimer or heterodimer made up of FOS (c-Fos, Fra-1, Fra-2) and JUN (c-Jun, JunB, JunD) protein family members<sup>30</sup>. AP-1 transcriptional factors are already clearly associated with *FLG* gene expression, as the *FLG* gene contains AP-1 Jun/Fos family sites in its regulatory regions, and a high basal level of *FLG* gene expression in keratinocytes depends on the AP-1 component of the c-Jun/c-Fos heterodimers<sup>31-33</sup>. Our results suggest that adiponectin regulates the activity of critical AP-1 components. We showed that the activity of c-Jun and Fra1 significantly increased after adiponectin treatment, and the effect was suppressed when MAPK signaling was inhibited by p38, JNK, and ERK inhibitors. Additionally, the effect of adiponectin on *FLG* expression was blocked by additional treatment with p38 and ERK inhibitors. Together, these results suggest that adiponectin stimulates *FLG* expression via a mechanism that depends on AP-1 transcriptional factors and MAPK signaling.

In addition, adiponectin exerted a synergetic effect with calcium on the induction of *FLG* expression. Accordingly, adiponectin might promote the differentiation of keratinocytes by affecting the level of intracellular calcium. The influence on differentiation could be considered one of the mechanisms associating adiponectin and *FLG* expression.

In conclusion, our findings suggest that adiponectin acts to regulate *FLG* expression. The present study demonstrated for the first time that adiponectin plays a role in regulating

the expression and processing of *FLG* in NHEKs. Therefore, adiponectin may be a potential therapeutic agent to control skin diseases that alter the skin barrier.

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## CONFLICTS OF INTEREST

The authors have nothing to disclose.

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