

## Inactivation of mitogen-activated protein kinase signaling pathway reduces caspase-14 expression in impaired keratinocytes

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### ABSTRACT

**Objective(s):** Several investigations have revealed that caspase-14 is responsible for the epidermal differentiation and cornification, as well as the regulation of moisturizing effect. However, the precise regulation mechanism is still not clear. This study was aimed to investigate the expression of caspase-14 in filaggrin-deficient normal human epidermal keratinocytes (NHEKs) and to explore the possible mechanism that contributes to the regulation of caspase-14.

**Materials and Methods:** The filaggrin-deficient NHEKs were induced by transfection with lentivirus (LV) vector encoding small hairpin RNAs (shRNA). The inhibitors SB203580, PD98059 and SP600125 were used for suppressing the expression of p38 mitogen-activated protein kinase (MAPK), p44/42 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). The expression of filaggrin, p38 MAPK, p44/42 MAPK and SAPK/JNK, caspase-14, keratin1 and keratin2 were detected by western blot.

**Results:** In filaggrin-deficient NHEKs, the expression of p38, p44/42 MAPK and SAPK/JNK and caspase-14 were significantly decreased. The inhibition of p38 and SAPK/JNK reduced the expression of caspase-14, while the p44/42 MAPK showed no consistent effects. Moreover, the filaggrin knockdown decreased the expression of keratin2, but had no effects on the level of keratin1.

**Conclusion:** The decreased expression of caspase-14 in filaggrin-deficient NHEKs may be induced by the inactivation of MAPK signaling pathway. These provide a novel perspective to understand the mechanism for the protective effects of filaggrin and caspase-14 on skin barrier function.

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### Introduction

The principal function of the human epidermal the stratum corneum (SC) is to establish and maintain an effective barrier function against dehydration or overly transepidermal water loss and against exogenously environmental insults (1). Defective epidermal differentiation and cornification have been observed in various skin disorders, such as the ichthyoses, psoriasis and atopic dermatitis (AD). Of them, AD has been characterized by pruritus, dry, desquamation, scaling erythematous papules and plaques due to defective barrier function caused by the abnormal epidermal differentiation and cornification (2-4). Moreover, the abnormal epidermal differentiation and cornification can further cause the damage of epidermal barrier function and promote the recurrence and aggravation of skin disorders. It can be seemed as a vicious circle. Therefore, it is highly paramount to explore the molecular mechanism underlying the

epidermal differentiation and formation of SC for developing potential therapies against skin disorders.

The SC is the end layer of terminal differentiation of keratinocytes in epidermis. During formation of SC, besides the involvement of keratins, several differentiation associated proteins uniquely are highly expressed in the terminally differentiated layers of the epidermis (5). For example, caspase-14 is a specially and highly expressed in the differentiating epidermis keratinocytes (6). Caspase-14, especially expressed in keratinocytes, is associated with terminal differentiation and cornification of epidermis keratinocytes to form the complete SC (7, 8). The well accepted mechanism is that caspase-14 can facilitate the process of dephosphorylation and proteolysis of profilaggrin to form filaggrin and further exhibit its protective effects on skin barrier (9, 10). Thus, we speculate that caspase -14 may play an essential role in the development of AD. However, the detailed

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information about the role of caspase-14 in AD is still deficient.

It has been reported that the activation of mitogen-activated protein kinase (MAPK) signaling pathway can accelerate the expression of caspase-14 in psoriasis (11). Our previous study indicated that the activation of P38 and c-Jun NH(2)-terminal kinase (JNK) pathway were inhibited in filaggrin-deficient normal human epidermal keratinocytes (NHEKs); and moreover, the inhibition of MAPK signaling pathway reduced the level of filaggrin (12). Filaggrin is a key molecular structure in skin barrier function and plays a pivotal role in terminal differentiation of the epidermis. The absence of filaggrin has been extensively reported to contribute to the dysfunction of skin barrier function and to be associated with the development of skin disorders (13-16). Our previous result found that the deficiency of filaggrin in NHEKs induced the decrease of cornified cell envelope related proteins including cytokeratin (CK)-5, 10, 14, loricrin, and involucrin (17). Therefore, we used the NHEKs with filaggrin deficient to simulate AD injury and further to uncover the underlying effect of caspase-14 in filaggrin-deficient NHEKs.

To address the problem, in filaggrin-deficient NHEKs, the level of p38 MAPK, p44/42 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and caspase-14 were investigated in this study. Furthermore, the effects of p38 MAPK, p44/42 MAPK, and SAPK/JNK inhibition on the expression of caspase-14 were also investigated. Hopefully, the results will provide novel insights for the underlying mechanism that is responsible for the dysregulation of caspase-14 in the development of AD.

## Materials and Methods

### Cell culture

NHEKs were purchased from Invitrogen and cultured in EpiLife medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calfserum (FCS, Gibco, Carlsbad, CA, USA), 1.5 mM L-Glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA, USA) on a 6 cm dish with 5% CO<sub>2</sub> at 37 °C. The culture medium was replaced twice a week.

### Knockdown of filaggrin by shRNA transfection

Based on the results of preliminary experiment, the small hairpin (shRNA) with best silencing effect was chosen for this study and sequenced as GTTGCTCAAGCATATTATT (position: nt-274). Scramble oligos and the target vector pGLV-H1-GFP (GenePharma Co. Shanghai, China) were digested with BamHI and EcoRI. The shRNA vector construction procedure and NHEKs transfection were performed as described in our previous study (12). The shRNA-infected cells were regarded as shRNA filaggrin group; cells infected with control shRNA unrelated to filaggrin sequence as negative

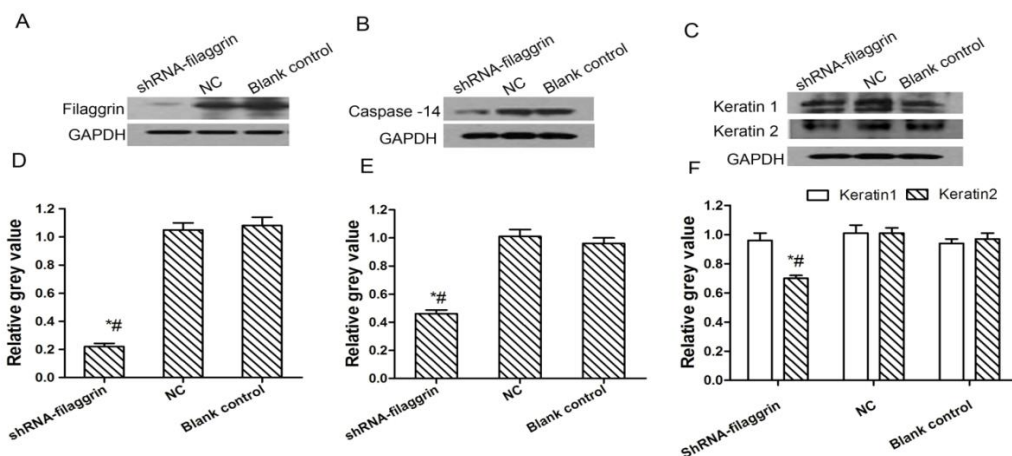
control (NC) group; and cells without infection treatment as blank control group. After cultured for 2 to 3 days, cells were subjected to western blot analysis.

### Western blot

The cells were harvested and the proteins were extracted with Mammalian Protein Extraction Reagent (M-PER, Pierce, Rockford, IL, USA), followed by thermal denaturation at 100 °C for 5 min. After cooling down, the supernatants were centrifuged at 12,000 rpm for 10 min at 4 °C to remove the insoluble precipitate. A total of 20 µl proteins was separated on 10% SDS-PAGE gels and transferred to a PVDF (Polyvinylidene Fluoride) membrane (Millipore, Bedford, MA, USA). After blocked with blocking buffer for 2 hr and washed with TBST (Tris-buffered saline containing Tween 20) for 3 times, the membranes were incubated with primary antibody overnight at 4 °C, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000) for 2 hr at room temperature. After incubated with SuperSignal West Pico Chemiluminescent Substrates (Pierce, Appleton, WI, USA), membranes were exposed to X-ray film and analyzed by Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the internal loading control. The primary antibodies used were listed as follow: Rabbit Anti-Filaggrin (Cat#PRB-417P, 1:250, COVANCE, Berkeley, CA, USA), p44/42 MAPK (#9926, 1:1, 000), SAPK/JNK (#9926, 1:1, 000), p38 MAPK (#9926, 1:250), Phospho-p44/42 MAPK (#9910, 1:250), Phospho-SAPK/JNK (#9910, 1:130), Phospho-p38 MAPK (#9910, 1:280, Cell Signaling, Beverly, MA, USA), Caspase-14 (#GTX111888, 1:20, GeneTex, Irvine, CA, USA), keratins1 (#16848-1-AP, 1:200), and keratins2 (#16849-1-AP, 1:200, Proteintech Group, Chicago, USA). The same experiment was repeated for 3 times.

### Inhibition of the p38 MAPK, p44/42 MAPK, and SAPK/JNK

NHEKs were seeded in 6 cm dish at a density of 5×10<sup>6</sup> cells and cultured in DMEM (dulbecco's minimum essential medium) for 24 hr with 5% CO<sub>2</sub> at 37 °C, followed by treatment with SB203580, PD98059 and SP600125 (Cell Signaling Technology, Beverly, MA, USA) at 120 µM, 200 µM, and 50 µM for inhibition of p38 MAPK, p44/42 MAPK, and JNK, and then incubated for 24 hr with 5% CO<sub>2</sub> at 37 °C. The concentrations of the inhibitors were determined in the preliminary experiments to achieve full inhibitory effects, and DMSO (dimethyl sulfoxide, 0.1%) was served as the solvent control. The expression of p38 MAPK, p44/42 MAPK, SAPK/JNK and caspase-14 were determined by western blot as described above. Each experiment repeated thrice.



**Figure 1.** The expression of caspase-14 and keratin 1, 2 by western blot in filaggrin-deficient normal human epidermal keratinocytes. A, D The knockdown efficiency of filaggrin at protein level. B, E The expression of caspase-14 in filaggrin-deficient NHEKs. C, F The expression of keratin 1, 2 in filaggrin-deficient NHEKs. \*indicated  $P < 0.05$  vs. negative group (NC), # indicated  $P < 0.05$  vs. blank control group. Each experiment repeated thrice

**Data analysis**

Data were presented as mean±standard deviation (SD). Data analysis was performed by one-way ANOVA using SPSS11.0 (SPSS Inc., Chicago, IL, USA). The differences between groups were compared using *Least Significant Difference (LSD)* test.  $P < 0.05$  was considered to be significantly different.

**Results**

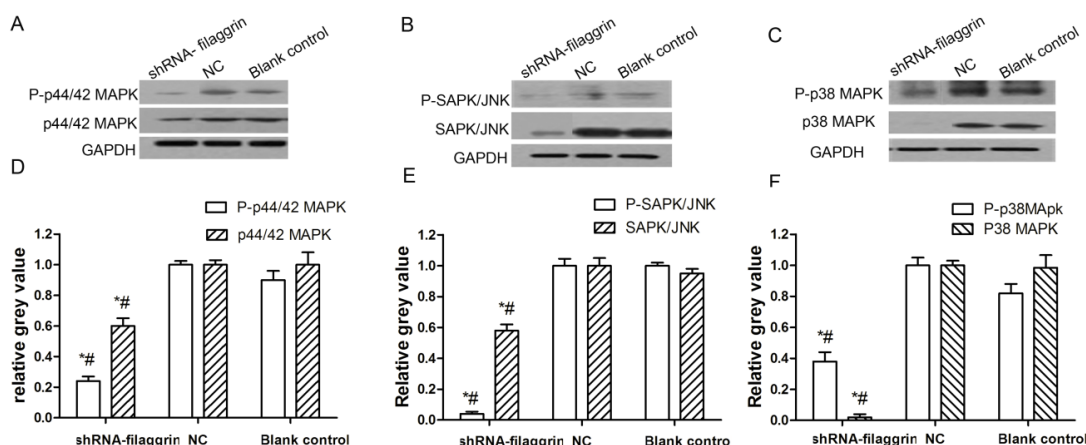
**The expression of keratins 1,2, caspase-14, p38, p44/42 MAPK and SAPK/JNK in filaggrin-deficient NHEKs**

After NHEKs were exposed to the lentivirus encoding shRNA of filaggrin, the knockdown efficiency of filaggrin at protein level (88%) was significantly lower than that in control and NC group (Figure 1A, D). Compared with the control and NC

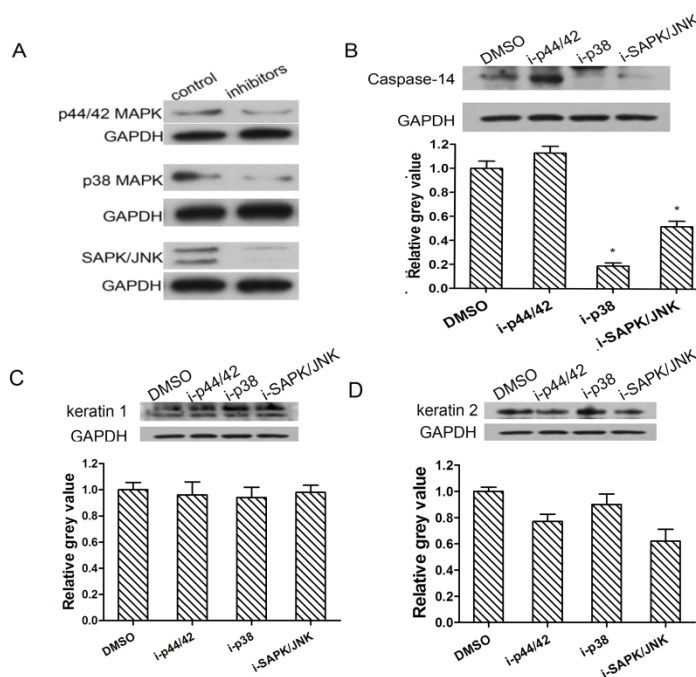
group, the expression of caspase-14 was significantly reduced in filaggrin-deficient NHEKs (Figure 1B, E). Moreover, the filaggrin knockdown resulted in the obvious decrease of keratins 2, but had no effects on the expression of keratins 1 (Figure 1C, F). After filaggrin knockdown, the expression of phosphorylated p38, p44/42 MAPK and SAPK/JNK were significantly lower than that in control and NC group (Figure 2).

**Effects of MAPK signaling pathway inhibition on the expression of caspase-14, and keratins 1,2**

The expression of p38, p44/42 MAPK and JNK were effectively inhibited by the corresponding inhibitors (Figure 3A). The inhibition of p38 and JNK significantly blocked the expression of caspase-14, while the p44/42 MAPK inhibition had no obvious



**Figure 2.** The expression of p44/42 mitogen-activated protein kinase (A,D), stress-activated protein kinase/c-Jun N-terminal kinase (B,E) and p38 (C, F) in filaggrin-deficient normal human epidermal keratinocytes. \* indicated  $P < 0.05$  vs. negative group (NC), # indicated  $P < 0.05$  vs. blank control group. P-p44/42 MAPK, P-SAPK/JNK, P-p38 indicated the phosphorylated p44/42 MAPK, SAPK/JNK and p38. Each experiment repeated thrice



**Figure 3.** The effects of inhibition of p44/42 mitogen-activated protein kinase, stress-activated protein kinase/c-Jun N-terminal kinase and p38 on the level of caspase-14 and keratin 1, 2. A: The inhibition efficiency of p44/42 MAPK, SAPK/JNK and p38. B: The expression of caspase-14 after inhibition of p44/42 MAPK, SAPK/JNK and p38. C The expression of keratin 1 after inhibition of p44/42 MAPK, SAPK/JNK and p38. D The expression of keratin 2 after inhibition of p44/42 MAPK, SAPK/JNK and p38.\* indicated  $P < 0.05$  vs dimethyl sulfoxide group. Each experiment repeated thrice

effects on the expression of caspase-14 (Figure 3B). The inhibition of p38, p44/42 MAPK and JNK reduced the expression of keratins 2, but presented no effects on the level of keratins 1 (Figure 3C, D).

## Discussion

Filaggrin and caspase-14 are the crucial markers related to the terminal differentiation of the epidermis and formation of SC. Moreover, the MAPK signaling pathway has been revealed to be involved in the regulation of keratinocyte differentiation and skin barrier function (18). Our previous study had indicated that the activation of MAPK signaling pathway was blocked in filaggrin-deficient NHEKs, and the inhibition of MAPK signaling pathway was related with the decreased expression of filaggrin (12). However, the level of caspase-14 in filaggrin-deficient NHEKs and relevant regulation mechanism are still not clear. Our results indicated that the level of caspase-14 was decreased in filaggrin-deficient NHEKs and was reduced by the inhibition of MAPK signaling pathway.

It has been well accepted that caspase-14 is associated with the processing of filaggrin monomers and the development of natural moisturizing factors of the skin. Caspase-14-deficient epidermis is characterized by reduced skin-hydration levels and increased water loss (19). Recently, several studies

have investigated the responsible factors for the regulation of caspase-14. Retinoic acid (20)

and glucocorticoid receptor (21) have been demonstrated to inhibit the expression of caspase-14. Vitamin D3 (22) or green tea polyphenol (11, 23) can up-regulate the expression of caspase-14. Moreover, the acceleration of caspase-14 is related to the activation of MAPK signaling pathway induced by green tea polyphenol (11). Similarly, in this study, the inhibition of p38 and SAPK/JNK significantly reduced the expression of caspase-14, but p44/42 MAPK inhibition showed no obvious effect on the caspase-14. It suggested that p44/42 MAPK might be involved in some other target gene regulation pathways, and further researches were needed to validate this hypothesis. The above results suggested that the inhibition of MAPK signaling pathway decreased the level of caspase-14, which might further result in the altered process of profilaggrin to form filaggrin, leading to the reduction of moisturizing factors and finally exacerbating the water loss of skin (24).

Previous studies have indicated the critical role of MAPK signaling pathways in regulating keratinocytes differentiation, proliferation and apoptosis (25-27). The activation of p38MAPK signaling pathway is consistently considered to promote the keratinocytes differentiation. However, some reports suggest that the activation of ERK1/2 or JNK exhibits negative effects on the keratinocytes differentiation (25, 28). For instance, was suggested that NHEK differentiation was essentially controlled by p38 activity, which might be negatively influenced by ERK1/2 activity (25). Also, it is shown that the inhibition of JNK in epidermal

keratinocytes was sufficient to initiate their differentiation program (28). Our preliminary results had revealed that filaggrin knockdown affected the epidermal terminal differentiation in NHEKs by decreasing the expressions of differentiation-related proteins. In the present study, the results indicated that the filaggrin knockdown significantly decreased the expression of p38, p44/42 MAPK and SAPK/JNK and blocked the activation of MAPK signaling pathway. These results suggested that some compensatory mechanisms might be involved in the process of keratinocytes differentiation. While the results of this study revealed that the expression of caspase-14 was also reduced in filaggrin-deficient NHEKs. Therefore, all the above results suggested that the inactivation of MAPK signaling pathway decreased the level of caspase-14 in filaggrin-deficient NHEKs.

Additionally, keratins are essential cellular skeleton proteins and keratin intermediate filaments can be aggregated by filaggrin into tightly aligned keratin bundles, which acts as a protein scaffold for the attachment of cornified envelope proteins and lipids that together form the stratum corneum (5, 29). In this study, we assessed the expression of keratin 1 and keratin 2 in filaggrin-deficient NHEKs and found that the deficiency of filaggrin had no impact on the keratin 1, but significantly decreased the expression of keratin 2. These results were consistent with the previous research that keratin 2 was expressed only in cells that also expressed filaggrin (5). It also suggested that filaggrin might be dispensable for keratin aggregation. Furthermore, we assessed the effects of MAPK inhibition on the keratin 1 and keratin 2, and the results presented that the inhibition of p38, p44/42 MAPK and SAPK/JNK reduced the expression of keratin 2, but had no effects on the level of keratin 1. These results suggested that the inactivation of MAPK signaling pathway in filaggrin-deficient NHEKs might influence the expression of keratin and further hinder the formation of SC. However, further research is needed to confirm the above conclusion.

### Conclusion

Our data suggest that the inactivation of MAPK signaling pathway in filaggrin-deficient NHEKs can decrease the expression of caspase-14, which may further aggravate the impaired skin barriers and the development of AD. Our work may provide a potential mechanism for the development of terminal differentiation of the epidermis and formation of SC involved with filaggrin and caspase-14.

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