Research Article

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MAPK pathway involved in epidermal terminal differentiation of normal human epidermal keratinocytes

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Abstract: Objective. To investigate the effect of mitogen-activated protein kinase (MAPK) signaling pathway in epidermal terminal differentiation.

Methods. The MAPK pathways (p38, ERK1/2, JNK) were inhibited by SB203580, PD98059, and SP600125 in normal human epidermal keratinocytes (NHEKs), respectively. Western blotting assays were performed to detect expression of filaggrin and differentiation-related proteins. The mRNA expressions of differentiation-related proteins were detected by real-time quantitative PCR (qRT-PCR).

Results. Inhibition of MAPK pathway by SB203580, PD98059, and SP600125 resulted in significant reduction of filaggrin expression in NHEKs. Inhibition of the p38 MAPK pathway decreased the expression of differentiation-related proteins (cytokeratin 5, cytokeratin 14, ST14, and SPRR3), Akt, and NF-κB. Inhibition of JNK also suppressed expression of cytokeratin 14, SPRR3, Akt, and NF-κB. However, inhibition of ERK1/2 merely decreased expression of SPRR3 and Akt.

Conclusion: MAPK pathways regulates epidermal terminal differentiation in NHEKs. The p38 signaling pathway plays an especially important role.

Keywords: Epidermal terminal differentiation; Keratinocyte differentiation; MAPK signaling pathway; Normal human epidermal keratinocytes (NHEKs); Differentiation-related proteins

1 Introduction

Skin is the first line of defense for the human body to resist environmental damage, water and nutrients loss, and prevent pathogens and allergens from entering. The stratum corneum (SC), the final product of terminal differentiation of keratinocytes in the epidermis, is the main element of epidermal skin barrier. A sophisticated signal transduction network controls keratinocyte physiology to ensure its normal function [1], and the mitogen-activated protein kinase (MAPK) signaling pathway occupies a central location [2]. The MAPK families have been proven to be essential for controlling diverse cellular behaviors, including cell proliferation, differentiation and apoptosis, for instance the p38 MAPK pathway, ERK1/2 MAPK pathway and JNK signaling pathway [3, 4]. Studies have shown that the MAPK signaling pathways integrate and mediate various signals and play a major role in regulating keratinocyte differentiation and the function of skin barrier [5-7]. The ERK1/2 signaling pathway has been shown to control keratinocyte differentiation; low ERK1/2 activity could induce keratinocyte differentiation and apoptosis [8]. The p38 and ERK pathways are activated and participate in keratinocyte differentiation among MAPK signaling pathways while the epidermal barrier is disrupted [8-11]. In addition, epidermal differentiation would be enhanced when JNK is inhibited by SP600125 [11].

This study aimed to investigate the role of MAPK signaling pathways in epidermal terminal differentiation based on the previous study.

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2 Materials and methods

2.1 Cell culture

NHEKs cells were purchased from Invitrogen and cultured in EpiLife medium (Invitrogen, Carlsbad, CA, USA) which was supplemented with 10% fetal calf serum (FCS, Gibco, Carlsbad, CA, USA), 1.5 mM L-Glutamine, 100 IU/mL penicillin, and 100 g/mL streptomycin (Gibco, Carlsbad, CA, USA). They were then maintained at 37 °C in 5% CO_2.

2.2 Inhibition of the MAPK pathways

Different concentrations of SB203580, PD98059, and SP600125 (Cell Signaling Technology, Beverly, MA, USA, dissolved in DMSO) were used to inhibit the p38 MAPK pathway, ERK1/2 pathway and JNK pathway according to the manufacturer's instructions. Approximately 5×10^6 NHEKs were seeded in 6-cm diameter dishes with DMEM medium at 37˚C with 5% CO_2 for 24 h. Then cells were divided into 3 groups as follows: i) treated with p38 pathway specific inhibitor (SB203580 at 5 μM, 10 μM and 20 μM), ii) treated with ERK1/2 pathway specific inhibitor (PD98059 at 50 μM, 100 μM, 200 μM), iii) treated with JNK pathway specific inhibitor (SP600125 at 50 μM, 100 μM, 200 μM), then incubated for 24 h. The appropriate concentrations of these three inhibitors were selected to achieve maximum inhibition effect and used in further experiments. Moreover, DMSO was used as control reagent and cells treated with DMSO as the control group. The expression level of filaggrin, p38, p-JNK, p-ERK1/2 and differentiation-related proteins were detected by Western blotting. This experiment was carried out in duplicate and independently repeated at least two times.

2.3 Western blotting analysis

Western blotting assays were used to detect expression of filaggrin and differentiation-related proteins. After 72h, NHEKs cells treated with inhibitor were collected to extract proteins and quantify using Mammalian Protein Extraction Reagent (M-PER, Pierce, Rockford, IL, USA). Each protein (20μL) was separated with 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with primary antibodies overnight at 4°C after blocking the membrane with 5% skim milk. After rinsing with TBST, the membrane was incubated with secondary antibody (Rabbit IgG, 1:2000, Cell Signaling, Beverly, MA, USA) at RT for 1 h. Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) was used to detect labeled protein with incubation of SuperSignal West Pico Chemiluminent Substrates (Pierce, Appleton, WI, USA). Then the labeled protein was exposured to X-ray film. Image J software was used to quantify the relative expression level of proteins by determining a ratio between the amount of them and GADPH. The primary antibodies were as follows: Filaggrin (1:250, Covance, Berkeley, CA, USA); Phospho-p38 MAPK (1:250, Cell Signaling, Beverly, MA, USA); Phospho-ERK1/2 (1:250, Cell Signaling, Beverly, MA, USA); Phospho-JNK (1:200, Cell Signaling, Beverly, MA, USA); Phospho-NF-κB (1:200, Cell Signaling, Beverly, MA, USA); Phospho-Akt (1:200, Cell Signaling, Beverly, MA, USA); Cytokeratin 5 (1:500, GeneTex, Irvine, CA, USA); Cytokeratin 14 (1:1000, GeneTex, Irvine, CA, USA); Loricrin (1:500, GeneTex, Irvine, CA, USA); SPRR3 (1:500, GeneTex, Irvine, CA, USA); and GAPDH (1:2000, Cell Signaling, Beverly, MA, USA).

2.4 Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was used to quantitatively detect mRNA expression of differentiation-related proteins. Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA by using the MMLV Reverse Transcriptase system (Promega, Madison, WI, USA). The Mx3000 Real-time PCR Instrument (Stratagen, La Jolla, CA, USA) was used for following real-time quantitative RT-PCR analysis with the program: 95°C for 3 min, 95°C for 30s, 62°C for 40 s, with a repetition of 40 cycles. Real-time RT-PCR data was analyzed by comparative CT (ΔΔCT) method. The expression level of mRNA was normalized to the endogenous control GAPDH. Primers sequences are shown in Table 1.

2.5 Statistical analysis

Experimental data were analyzed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA) software with one-way ANOVA and the Student's *t*-test to analyze differences between groups. Quantitative data was presented as the mean ± standard deviation (n = 3). A value of $P < 0.05$ for the difference was considered statistically significant.

Table 1: Primer Sequences used in RT-PCR

Gene	Primer Sequence
Cytokeratin 5[12]	Upstream: 5´- ATCGCCACTTACCGCAAGCTGCTG-3´
	Downstream: 5´- GAGGGAAACACTGCTTGTGACAACAGAG -3´
Cytokeratin 14 [13]	Upstream: 5´- CCGACACCTTCTCTTCACTCA -3´
	Downstream: 5´- AGGAGCCCTTCATGGAGCTG -3´
Loricrin [14]	Upstream: 5´- ACGTCTCCTCGCAGCAGG -3´
	Downstream: 5´- CTATTTGGACGGCCAGGT -3´
ST14	Upstream: 5´- CACTGGTGGTTCTACTGAC -3´
	Downstream: 5´- GTTTTCCAGGGTCCTCCGA -3´
SPRR3 [15]	Upstream: 5´- CTTCTCTGCACAGCAGGRCC -3´
	Downstream: 5´- AGCAATTTAATGAGGGAAGAGC -3´
filaggrin	Upstream: 5´-CACAAGATTCTGCGTATCACTCAGG-3´
	Downstream: 5´- GCCTTTCAGTGCCCTCAGATTG-3´
GAPDH	Upstream: 5´- CATGAGAAGTATGA CAACAGCCT-3´
	Downstream: 5´- AGTCCTTCCACGATA CCAAA GT-3´

3 Results

3.1 Inhibition of MAPK decreased the expression of filaggrin in NHEKs

To examine whether MAPK pathways had an effect on filaggrin expression, we cultured NHEKs cells with a specific inhibitor (SB203580) of the p38 MAPK pathway, a specific inhibitor (PD98059) of ERK1/2 pathway, and a specific inhibitor (SP600125) of JNK pathway. The data showed that the p38 MAPK pathway, ERK1/2 pathway and JNK pathway were inhibited by SB203580, PD98059 and SP600125, respectively, and that the 20 μM, 100 μM and 50 μM concentrations inhibited to maximum effect, respectively. These concentrations were used in further experiments (Fig.1A-C). The expression of filaggrin protein was reduced when the MAPK pathway was inhibited (Fig.1A-C). Moreover, the expression of filaggrin mRNA was decreased by inhibiting the MAPK pathway (Fig.1 D). Thus, this finding suggested that it was the inhibition of the p38 MAPK pathway, ERK1/2 pathway and JNK pathway that down-regulated filaggrin in NHEKs.

3.2 Inhibition of MAPK signaling pathway affected expression of differentiation-related proteins in NHEKs

In order to further investigate whether the inhibition of MAPKs affected NHEKs differentiation, we measured the expression of differentiation-related proteins in NHEKs. The expressions of Cytokeratin 5, Cytokeratin 14 and ST14 were significantly decreased compared with the control group in NHEKs (P<0.05) after treatment with the p38 MAPK pathway specific inhibitor, and in particular, the expression of p-Akt, p-NF-κB and SPRR3 was remarkably reduced (P<0.01) (Fig. 2A, B). However, treatment with the ERK1/2 pathway specific inhibitor not only decreased the expressions of SPRR3 and p-Akt (P<0.05), but also enhanced the expression of Cytokeratin 14, locrin and ST14 (Fig. 2A, B). In addition, the specific inhibition of SP600125 on the JNK pathway only affected the expression of SPRR3, p-Akt, p-NF-κB and ST14, in that SPRR3, p-Akt, and p-NF-κB were inhibited (P<0.05), while ST14 expression was promoted (Fig. 2A, B). The qRT-PCR results showed similar results; inhibition of p38 MAPK significantly decreased mRNA expression of Cytokeratin 5, Cytokeratin 14 and SPRR3 (Fig. 3). These data revealed that the p38 MAPK pathway, ERK1/2 pathway and JNK pathway have diverse functions in NHEK epidermal differentiation.

4 Discussion

Filaggrin is a vital component of keratohyalin granules and the skin barrier [16], and is generally considered as one of the major markers of epidermal terminal differentiation and formation of the SC. Kezic et al found that epidermal filaggrin and its degradation product levels were reduced or completely lost when the *FLG* gene was mutated [17]. Loss of filaggrin can result in serious defects in the epithelial barrier, incomplete keratin accumulation, and loss of transepidermal water [18, 19]. In this report, it was revealed that inhibition of p38 MAPK, ERK1/2, and JNK pathways down-regulated expression of filaggrin

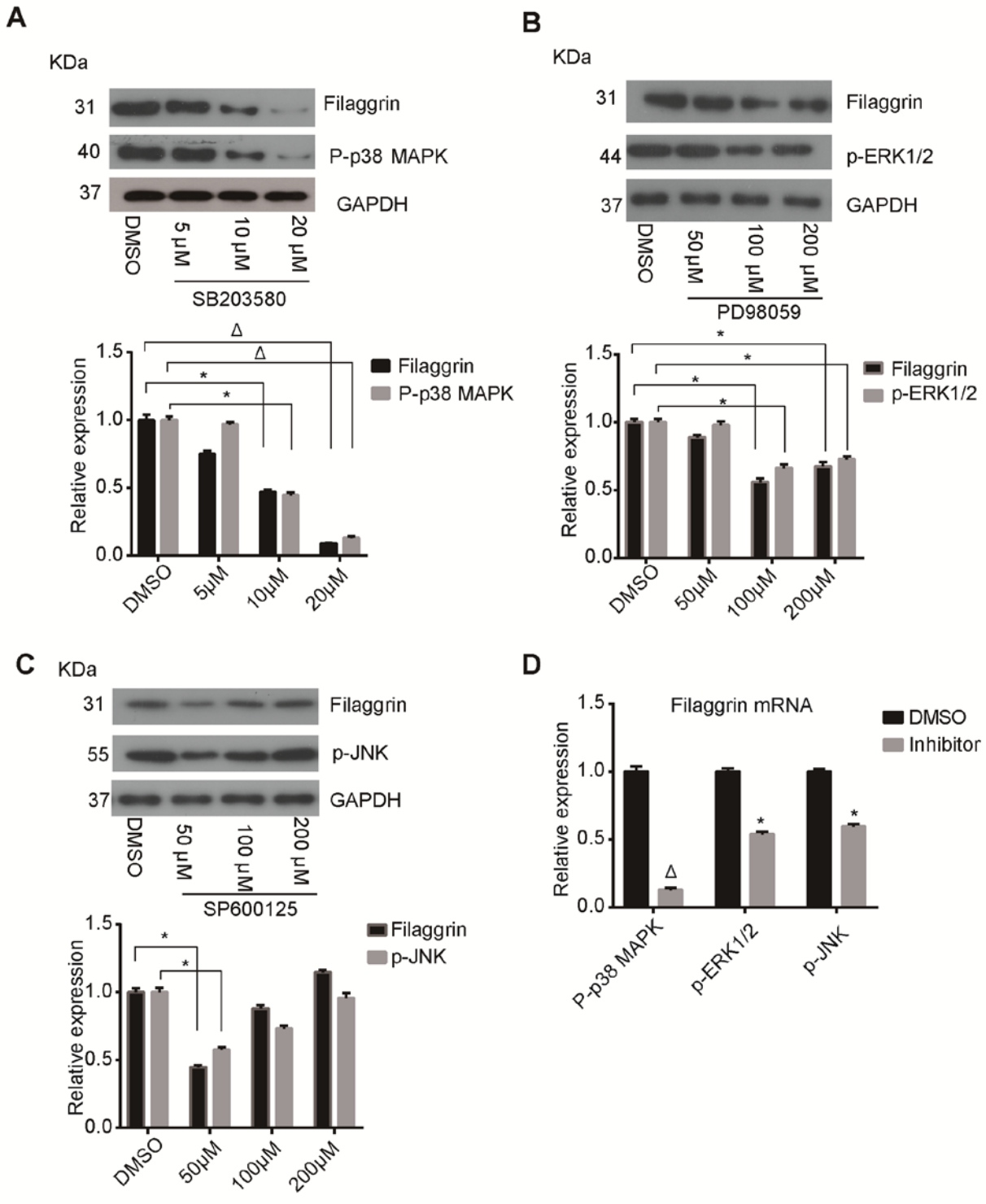

Figure 1: Inhibition of the p38, ERK1/2 and JNK pathways decreased the expression of filaggrin. A. Western blotting results of filaggrin (38 k Da), and p38. NHEKs were treated with SB203580 (p38 inhibitor) at 5 μM, 10 μM and 20 μM for 24 h. DMSO treatment was used as control B. Western blotting results of filaggrin (38 k Da), and ERK1/2. NHEKs were treated with PD98059 (ERK1/2 inhibitor) at 50 μM, 100 μM and 200 μM for 24 h. DMSO treatment was used as control. C. Western blotting results of filaggrin (38 k Da), and ERK1/2. NHEKs were treated with SP600125 (JNK inhibitor) at 50 μM, 100 μM and 200 μM for 24 h. DMSO treatment was used as control. D. qRT-PCR results of expression of filaggrin mRNA in NHEKs cells treated with 20 μM SB203580, 100 μM PD98059 or 50 μM SP600125, respectively. n = 3, $^{*}P < 0.05$, $^{\Delta}P < 0.001$.

in NHEKs, suggesting that the MAPK pathway may have effect on terminal differentiation of NHEKs.

The MAPK signaling pathway has been shown to play critical roles in epidermal differentiation and skin barrier function [20-23]. The MAPK signaling pathways can integrate and mediate various signals involved in keratinocyte differentiation, including epidermal growth factor receptor (EGFR), integrin and calcium signaling. Inhibition of the p38 MAPK pathway in keratinocytes could interfere with keratinocyte differentiation. Thus, we investigated the role of the MAPK signaling pathway in epidermal differentiation of NHEKs through detecting expression of differentiation-related proteins. Epidermal proliferation of normal skin is limited to the basal layer, and is related to specific keratin proteins, such as Cytokeratin 5 and Cytokeratin 14 [24]. Researchers have confirmed that Cytokeratin 5 and Cytokeratin 14, which are expressed in the basal layer, are related to cell differentiation, and contribute to maintain the cell proliferation potential [25, 26]. In addition, small proline-rich protein (SPRRs) and loricrin are also associated with increased epithelial proliferation. Loricrin is a major cornified cell envelope (CE) protein that forms the outermost layer of epidermis and plays an important role in maintaining the epidermal barrier. ST14 is generally known to be involved in cell growth and differentiation, and is suggested to be related to the regulation of differentiation in suprabasal keratinocytes [27]. In this study, we found that inhibition of JNK, ERK1/2, and p38 MAPK pathways resulted in significant reduction of SPRR3, but had different effects on expression of Cytokeratin 14, loricrin, and ST14 (Fig. 2). Thus, it suggested that MAPK pathways affected terminal differentiation of NHEKs via regulating differentiation-related proteins. In addition, inhibition of MAPK signaling pathway suppressed activation of Akt and NF-κB pathway (Fig. 2). NF-κB and Akt have also been revealed to play an important role in epidermal differentiation[28, 29]. These signaling pathways are interrelated with each other in cellular activities, and certain forms of cellular stress could enhance p38 phosphorylation, also leading to activation of the NF-κB pathway in human keratinocytes[30]. Furthermore, Oh et al found that activation of NF-κB induced by tumor necrosis factor α (TNF-α) impacts the activation of Akt in human pulmonary epithelial cells [31]. Hence, we inferred that the mechanisms of NF-κB and Akt action in epidermal differentiation might be closely associated with MAPK pathways.

In this study of the MAPK signaling pathway in epidermal differentiation, we found that JNK pathway, ERK1/2 pathway, and p38 MAPK pathway were associated with the regulation of differentiation-related proteins in NHEKs (Fig.2), which was consistent with previous studies [32]. However, the three pathways were different in regulating the differentiation-related proteins (Fig. 2). Inhibition of the p38 MAPK signaling pathway led to the significantly decreased expression of Cytokeratin 5, Cytokeratin 14 and ST14, and the expression of p-Akt, p-NF-κB, SPRR3 reduced remarkably. But only SPRR3 and p-Akt expressions were inhibited slightly in the case of ERK1/2 signaling pathway inhibition, while expression of Cytokeratin 14, locrin and ST14 increased remarkably, indicating there might be a feedback compensatory mechanism to connect pathways. However, the inhibition of JNK signaling pathway only affected the expres-

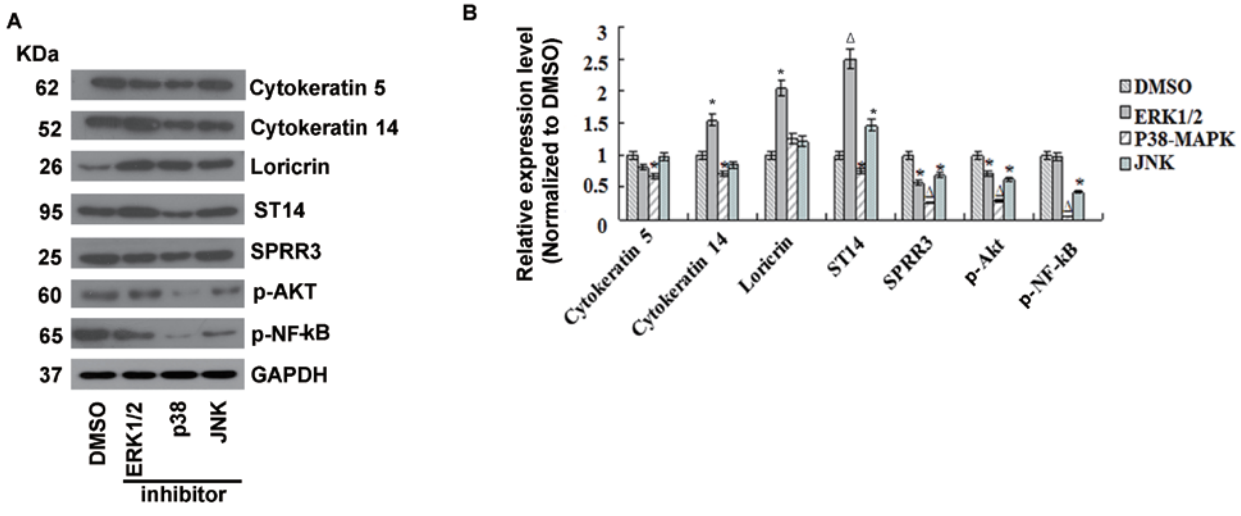

Figure 2: Inhibition of the MAPK pathway affected expression of differentiation-related proteins. A. Western blotting results of differentiation-related proteins. NHEKs were treated with 20 μM SB203580, 100 μM PD98059 or 50 μM SP600125, respectively. B. Semi-quantitative analysis of differentiation-related proteins, compared with the control (DMSO), n = 3, *P < 0.05, ΔP < 0.001.

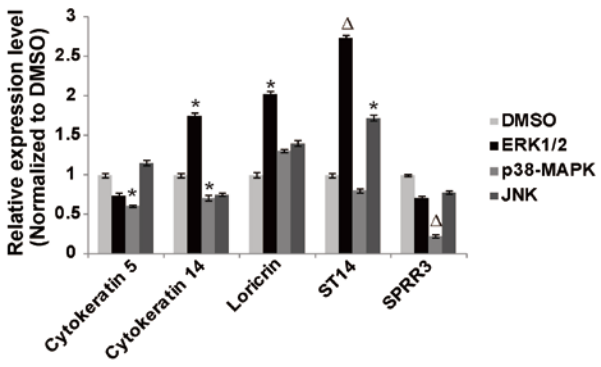

Figure 3: Inhibition of the MAPK pathway affected mRNA expression of differentiation-related proteins. qRT-PCR results of mRNA expression of differentiation-related proteins. NHEKs were treated with 20 μM SB203580, 100 μM PD98059 or 50 μM SP600125, respectively. n = 3, * $P < 0.05$, Δ$P < 0.001$.

sion of SPRR3, p-Akt, p-NF-κB and ST14, in which SPRR3, p-Akt and p-NF-κB were inhibited, while ST14 expression was enhanced. These results demonstrated that the JNK pathway, ERK1/2 pathway and p38 MAPK pathway might have different mechanisms to regulate epidermal differentiation in NHEKs. ERK1/2 exerted opposite effects of p38 in epidermal keratinocytes, which was consistent with Gazel A's study [33]. This experiment also suggested that expressions of p-Akt and p-NF-κB were less when p38 was inhibited than in the other cases when the JNK pathway or ERK1/2 pathway were inhibited, indicating that the MAPK p38 pathway may affect NHEKs differentiation through Akt and NF-κB. Furthermore, there was no significant change of NF-κB activation when ERK1/2 was inhibited. Previous studies have shown that there was no connection in induction of IL-6 between ERK1/2 and NF-κB in cardiac fibroblasts[34]. But Jiang B found that ERK1/2 has an effect on the regulation of NF-κB activation in vascular smooth muscle cells [35]. It might be different cell types and experimental models which cause these conflicting results. Therefore, further research is needed to explain and confirm the conflicting results.

5 Conclusion

Our previous study demonstrated that, in filaggrin-deficient NHEKs, the expression of p38, p44/42 MAPK and SAPK/JNK and caspase-14 were significantly decreased [36]. In conclusion, our data suggested that Filaggrin might regulate the expression of differentiation-related proteins via p38 MAPK pathway. Meanwhile, p38 MAPK pathway activation regulated the expression of filaggrin in NHEKs. The inhibition of the p38 MAPK pathway, which was regulated by knock-down filaggrin, affected downstream NF-κB and Akt pathways. In addition, the ERK pathway and JNK pathway also played a role in the keratinocyte differentiation process. Our study provided a potential mechanism for terminal differentiation deficiency in diseases related to filaggrin functional absence.

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Conflicts of interest: The authors have no conflicts of interest to declare.

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