Knock-down of filaggrin influences the mitogen-activated protein kinases signaling pathway in normal human epidermal keratinocytes

Shumei Wang[1], Liyun Qiu[2], Xianguang Meng[3], Ningning Dang[3]*

> Background: Filaggrin is an essential structural protein of the stratum corneum binding to the keratin intermediate filaments to form a dense protein-lipid matrix. However, the function of filaggrin in epidermal terminal differentiation is not completely understood.
Aim: To evaluate the effects of filaggrin on normal human epidermal keratinocytes (NHEKs) and to investigate the relevant mechanisms.
Methods: Short hairpin RNA (shRNA) technology was used to knock-down filaggrin in normal human epidermal keratinocytes (NHEKs). Western blot and real-time quantitative PCR (qRT-PCR) were performed to detect expression of filaggrin, differentiation-related proteins and MAPK-related proteins.
Results: Filaggrin was successfully knocked down in NHEKs (99% efficiency). We found that the lack of filaggrin significantly decreased the expression of some differentiation-related proteins, including Cytokeratin 5 protein, Cytokeratin 14 protein, ST14 protein and SPRR3 protein (P<0.05). In addition, filaggrin knock-down significantly decreased expression of p-p38, p-ERK1/2, p-JNK, p-Akt, and p-NF-κB in NHEKs.
Conclusion: Our study shows that filaggrin regulates epidermal terminal differentiation and impairs MAPK signaling pathway in normal human epidermal keratinocytes. <

Key words: differentiation-related proteins, epidermal terminal differentiation, keratinocyte differentiation, mitogen-activated protein kinases, normal human epidermal keratinocytes (NHEKs).

[1]Department of Community Medicine,
[2]Department of Pharmacy,
[3]Department of Dermatology,
Jinan Central Hospital affiliated to Shandong University, No.105 Jiefang Road, Jinan 250013, Shandong Province, China.
Corresponding author: Ningning Dang
ningningdangxj@sina.com

Introduction

Skin is the first line of defense to protect the human body from environmental damage, loss of water and nutrient and entry of pathogens and allergens. The stratum corneum (SC) is the main component of the epidermal skin barrier, which is the final product of terminal differentiation of keratinocytes in epidermis.

The filaggrin gene (FLG) is mainly expressed in the granular layer of the skin, and the encoding profilaggrin protein is matured to filaggrin. Filaggrin is an important structural protein in the stratum corneum. It can enhance the terminal differentiation of the epidermis and the formation of skin barrier [1]. Mutations in FLG gene can result in reduced or complete loss of epidermal filaggrin and its degradation products [2]. The absence of filaggrin can lead to epithelial barrier defects, incomplete accumulation of keratin and loss of trans-epidermal water [3, 4]. FLG mutations are closely related to some common dermatological diseases, such as atopic dermatitis (AD) and ichthyosis vulgaris (IV) [5], but also to type 2 diabetes and cardiovascular and cerebrovascular diseases [6]. However, the roles of filaggrin in epidermal terminal differentiation is not fully understood.

In the present study, the function of filaggrin in human epidermal keratinocyte differentiation was studied. We used shRNA transfection to knock down the filaggrin in normal human epidermal keratinocytes (NHEKs). RT-PCR and Western blot were performed to detect the expression of differentiation-related proteins.

Materials and methods

Cell culture

NHEKs were obtained from Invitrogen (Carlsbad, CA, USA). They were cultured with EpiLife medium containing 10% fetal calf serum (FCS, Gibco, Carlsbad, CA, USA), 1.5 mM L-Glutamine, 100 IU/mL penicillin and 100 g/mL streptomycin (Gibco) in 6 cm dishes, at 37°C with 5% CO_2. The medium was refreshed twice a week.

Knockdown of filaggrin using shRNA transfection

We designed and synthetized four sequences of filaggrin shRNA, as follows: shRNA 1 (at nt-274, GTTGGCTCAAGCATATTATTT), shRNA 2 (at nt-769, CACCACTGATAGT CTATTATT), shRNA 3 (at nt-1627, CCACGAGCAATCGG-TAAATTT), shRNA 4 (at nt-4936, GTCCCATCAAGAAGATAGATT). *Bam*HI and *Eco*RI were used to cut the sequences of filaggrin shRNA, scramble oligos and target vector pGLV-H1-GFP (GenePharma Co. Shanghai, China). T4 DNA Ligase was used to ligate the vector with insert after gel purification. The ligation products (pGLV-FLG-GFP and pGLV-scr-GFP) were then sequenced. 293T cells were used for transfection with pGLV-FLG-GFP or pGLV-scr-GFP to generate the corresponding viruses which were used to infect NHEKs. The plasmid pHelper 1.0 and pHelper 2.0 were packaged based on standard protocols. When NHEKs attained to 90% confluence, a mixture of 0.5 µL lipofectamine 2000 (Invitrogen), 10 pmol siRNA and 75 µl OPTI-MEM medium (Invitrogen) were added to each dish and incubated for 20 min at room temperature (RT). After changing culture medium to serum-free DMEM in each dish, infected cells were cultured for 72 h and then collected for RT-PCR and Western blot analyses.

Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was performed to quantitatively estimate the mRNA expression of filaggrin and measure the filaggrin knock-down efficiency. Total RNA from cultured NHEKs was isolated using Trizol reagent (Invitrogen) according to the published method. The purified RNA was reverse transcribed to cDNA by using the MMLV Reverse Transcriptase system (Promega, Madison, WI, USA) according to the manufacturer's instruction. Mx3000 Real-time PCR Instrument (Stratagen, La Jolla, CA, USA) was used to analyze quantitative RT-PCR of filaggrin according to the manufacturer's instruction. Primer sequences were as follows: Filaggrin: forward: 5'-CACAAGATTCTGCGTATCAC TCAGG-3', reverse: 5'-GCCTTTCAGTGCCCTCAGATTG-3'; K5: forward: 5'-ATCGC CACT-TACCGCAAGCTGCTG-3', reverse: 5'-GAGGGAAACACTGCTTGTGACAACA GAG-3'[7]; K14: forward: 5'-CCGACACCTTCTCTTCACTCA-3', reverse: 5'-AGGAG CCCTTCATGGAGCTG-3'[8]; Loricrin: forward: 5'-ACGTCTCCTCGCAGCAGG-3', reverse: 5'-CTATTTGGACGGCCAGGT-3'[9]; ST14: forward: 5'-CACTG-GTGGTTCTA CTGAC-3', reverse: 5'-GTTT TCCAGGGTCCTCCGA-3'; SPRR3: forward: 5'-CTTCTC TGCACAGCAGGRCC-3', reverse: 5'-AG CAATTTAATGAG-GGAAGAGC-3'[10]; GAPDH: forward: 5'-CATGAGAAGTATGACAACAGC CT-3', reverse: 5'-AGTCCTTC CACGATACCAAAGT-3'. The reaction system was: 2×Real-time PCR Master Mix 10 µL, 20 µM primers 0.2 µL, cDNA template 2 µL, 5 U/µl rTaq DNA polymerase 0.4 µL and ddH_2O 7.4 µL. The following PCR condition were used: 95 °C for 3 min, 95 °C for 30 s, 62 °C for 40 s, 40 cycles. This experiment was carried out in triplicate and independently repeated at least three times. The expression levels of filaggrin were normalized to an endogenous control, GAPDH. PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide for visualization.

Western blotting analysis

Western blotting was adopted to test the knock-down efficiency of filaggrin and the expression of differentiation-related proteins. Seventy-two hours after transfection, NHEKs cells were collected, proteins were extracted and quantified using the Mammalian Protein Extraction Reagent (M-PER, Pierce, Rockford, IL, USA). After 10% SDS-PAGE, proteins (20 µL) were transferred to PVDF membrane (Millipore, Bedford, MA, USA), and the non-specific binding sites of the membrane were blocked with 5% skim milk. Then the membrane was incubated with primary antibodies overnight at 4 °C. After rinsing with TBST, the membranes were incubated with HRP-conjugated secondary antibody (rabbit IgG, 1:2000, Cell Signaling, Beverly, MA, USA) at RT for 1 h. Labeled protein bands were detected using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) after incubation with SuperSignal West Pico Chemiluminent Substrates (Pierce, Appleton, WI. USA) and exposed to X-ray film. The Western blotting was performed three times. The quantification analyses were performed using Image J. The relative expression level of proteins was calculated by determining a ratio between their amount and that of GADPH. The rabbit polyclonal antibodies were : Filaggrin (1:250, Covance, Berkeley, CA, USA); Cytokeratin 5 (1:500,); Cytokeratin 10 (1:500); Cytokeratin 14 (1:1000); Loricrin (1:500); ST14 (1:500); SPRR3 (1:500), all from GeneTex (Irvine, CA, USA); Phospho-p38 MAPK (1:250,); Phospho-ERK1/2 (1:250); Phospho-JNK (1:200); Phospho-NF-κB (1:200); Phospho-Akt (1:200); GAPDH (1:2000), all from Cell Signaling (Beverly, MA, USA) .

Statistical analysis

The SPSS11.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the experimental data by one-way ANOVA. Quantitative data are presented as the mean ± standard deviation (n=3). The Student's t-test was used to analyze the difference between groups. $P < 0.05$ was considered as statistically significant.

Results

Knock-down efficiency of filaggrin for different shRNAs

In order to detect the knock-down efficiency of filaggrin by shRNA transfection, filaggrin-encoding mRNA
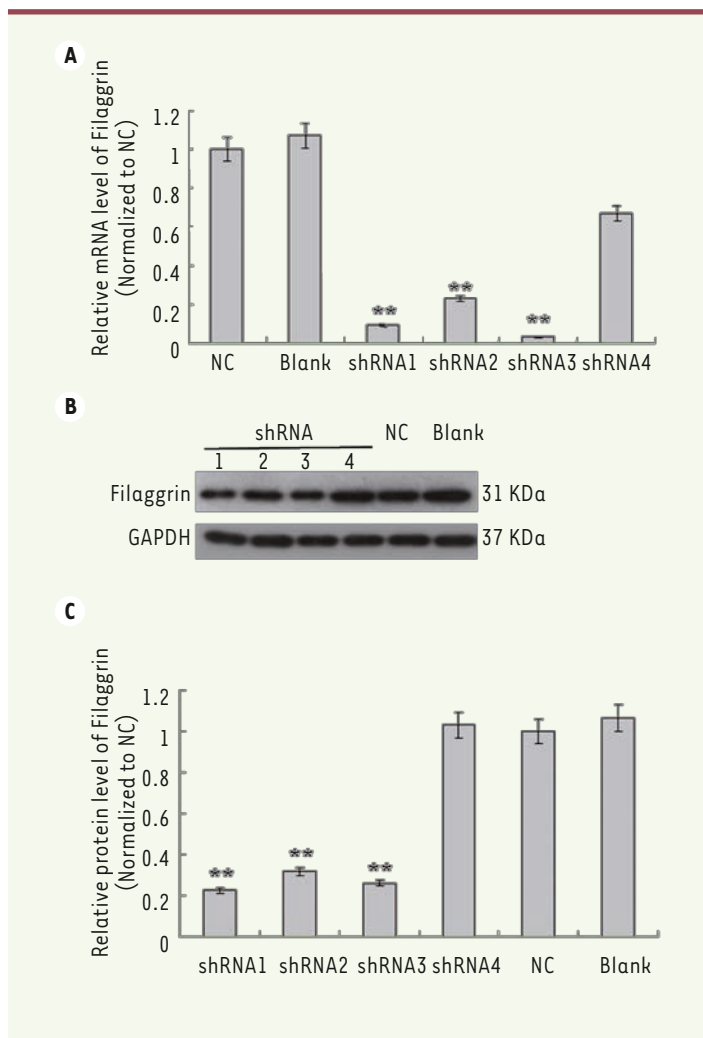
Figure 1. *Four shRNAs inhibited the filaggrin expression both at the mRNA and protein levels 72 h after shRNA transfection into NHEKs.* **A.** Expression of filaggrin mRNA after transfection with each kind of shRNA, compared to the control (NC). **B.** Western blotting of filaggrin after NHEKs transfection with each kind of shRNA. **C.** Semi-quantitative analysis of filaggrin protein levels for each kind of shRNA transfection into NHEKs, compared to the control group (NC). n=3, ** *P*<0.001.

and filaggrin were detected by RT-PCR analysis and Western blotting, respectively. The four different shRNAs treatments both decreased mRNA expression, with a knock-down efficiency of 99% (P<0.01), 77% (P<0.01), 97% (P<0.01) and 33% compared with the NC group, respectively (*Figure 1A*). The knock-down efficiency of filaggrin protein was 88% (P<0.01), 69% (P<0.01), 84% (P<0.01) and 0%, respectively (*Figure 1B, C*). Therefore, we selected the shRNA1 which had the most knock-down efficiency for use in the subsequent experiments.

Filaggrin knock-down in NHEKs inhibits cell differentiation

To investigate the function of filaggrin in NHEKs differentiation, we evaluated the expression of epidermis differentiation-related

proteins by Western blotting, including cytokeratin 5 (K5), cytokeratin 14 (K14), ST14 (matriptase), SPRR3 (small proline-rich protein 3) and loricrin (*Figure 2*). Filaggrin knock-down resulted in a significant decrease in K5, K14, ST14 proteins (P<0.05) and especially in SPRR3 (P<0.01), while no significant changes in loricrin content was observed. The result suggested that filaggrin knock-down inhibits NHEKs differentiation. qRT-PCR analysis showed similar results, the filaggrin knock-down significantly reducing the NHEKs content in K5, K14, ST14, and SPRR3 mRNA (P<0.05, *Figure 2C*).

Filaggrin knock-down reduces MAPK pathway signaling activation in NHEKs

To further examine whether filaggrin knock-down affected the Akt, NF-κB and MAPK pathways in NHEKs, Western blotting was performed to analyze the phosphorylation level of Akt, NF-κB, p38, ERK1/2 and JNK (*Figure 3*). The phosphorylation levels of p38 protein, ERK1/2 protein, JNK protein, Akt protein and NF-κB protein were decreased significantly (P<0.01) when filaggrin was knocked down, with the phosphorylation of Akt and JNK being almost completely blocked. These results indicate that filaggrin knock-down strongly impact the MAPK pathways and the Akt, and NF-κB signaling activation.

Discussion

Human skin consists of two different main layers, epidermis and subjacent dermis. The barrier of the epidermal skin-stratum corneum (SC) is the final product of the terminal differentiation of keratinocytes in epidermis. This terminal differentiation is strictly regulated by the sequential expression of various genes, a complicated multistep process [11]. Although studies have reported that many genes are involved in keratinocyte differentiation, there are also several important molecules that remain to be determined [12].

It is widely considered that filaggrin is one of the major markers for the epidermal terminal differentiation and formation of SC. However, the relevant regulation mechanism of filaggrin in epidermal terminal differentiation is not completely clear. The epidermal proliferation of normal skin is limited to the basal layer and related to certain keratin proteins, such as K5 and K14 [13]. The K5/K14 pair is expressed in basal layer, maintains the cell proliferation potential, and its expression is related to cell differentiation [14, 15]. In addition, the small proline-rich protein
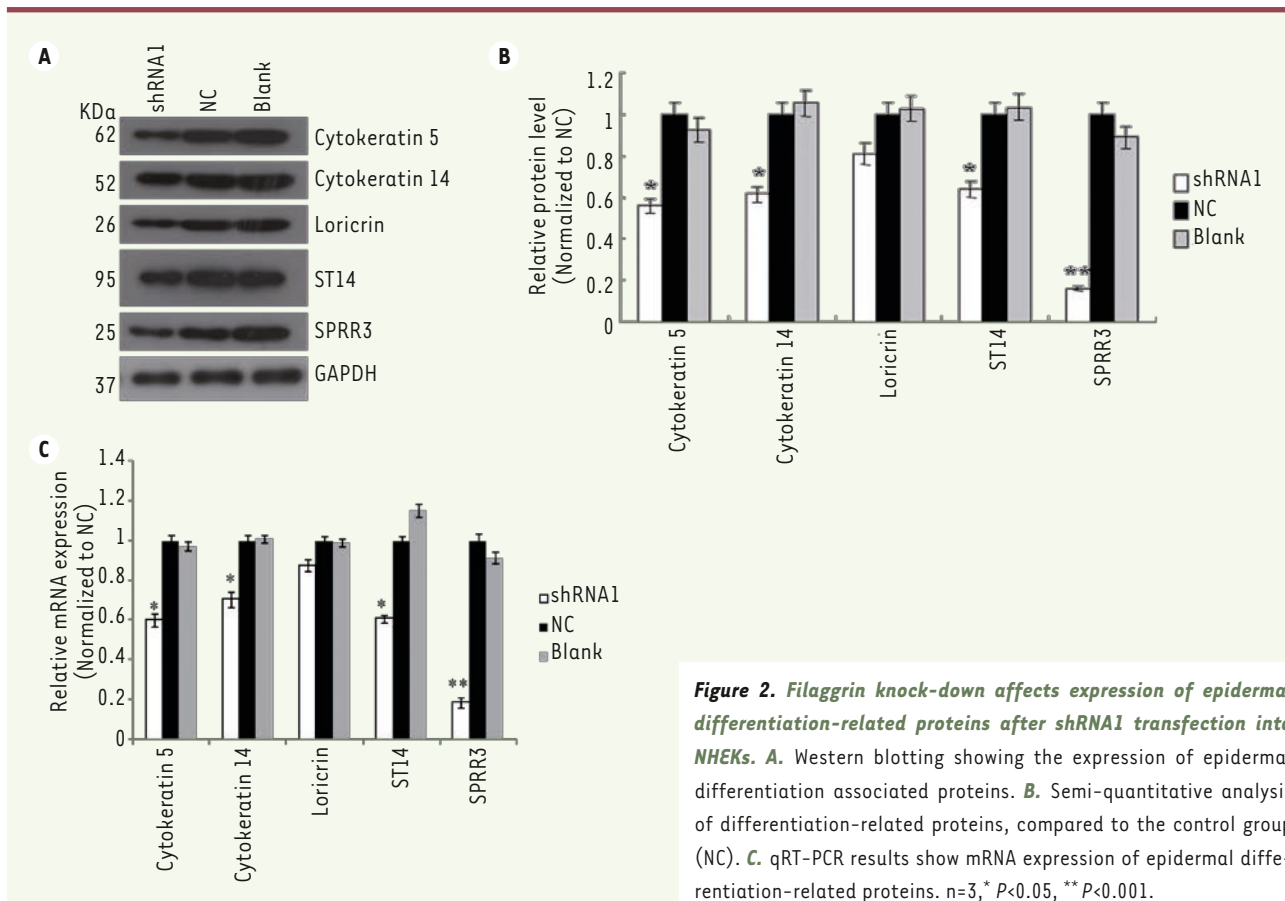
Figure 2. Filaggrin knock-down affects expression of epidermal differentiation-related proteins after shRNA1 transfection into NHEKs. A. Western blotting showing the expression of epidermal differentiation associated proteins. B. Semi-quantitative analysis of differentiation-related proteins, compared to the control group (NC). C. qRT-PCR results show mRNA expression of epidermal differentiation-related proteins. n=3,* P<0.05, ** P<0.001.

(SPRRs) are associated with increased epithelial proliferation, in particular a major cornified cell envelope (CE) protein, loricrin. The CE forms the outermost layer of the epidermis and maintains the epidermal barrier. Several studies have suggested that ST14 is involved in cell growth and differentiation and may be associated with the regulation of the suprabasal keratinocyte growth and differentiation [16]. In the present study, Western blotting results showed that filaggrin knock-down reduced K5, K14, ST14, loricrin, and SPRR3 expressions in NHEKs, indicating that filaggrin knock-down had an inhibitory effect on epidermal terminal differentiation of NHEKs through the down-regulation of differentiation-related protein expression *(Figure 2)*.

The mitogen-activated protein kinases (MAPK) signaling pathway has been revealed to play critical roles in epidermal differentiation and skin barrier function. It involves p38 MAPK, ERK1/2 MAPK and JNK signaling pathways [17-20]. Lack of ERK1/2 caused a decrease in the expression of terminal keratinocyte differentiation genes [21-23]. Besides, another signaling pathway involving NF-κB and Akt has been also shown to play an important role in epidermal differentiation [24, 25]. These different signaling pathways are not independent in cell. Cellular stress can promote p38 phosphorylation, leading to the activation of the NF-κB pathway in human keratinocytes [26]. Moreover, it has been suggested that the NF-κB activation induced by TNF-α affect the activation of Akt [27]. Hence, our data suggest that NF-κB and Akt are intervening in epidermal differentiation in a way closely associated with the MAPK pathways. We found that the phosphorylation levels of p38, ERK1/2, JNK, Akt and NF-κB are inhibited significantly by the loss of filaggrin, indicating that filaggrin knock-down can inhibit the activation of MAPK, NF-κB and Akt signaling pathways *(Figure 3)*.

Conclusion

In summary, this study demonstrates that filaggrin knock-down can inhibit the differentiation of NHEKs by down-regulating the expression of K5, K14, ST14 and SPRR3. In addition, it reduces the activation of multiple signaling pathways, including MAPK, NF- kappa B and Akt pathways. However, whether the intermediate filament-related proteins are regulated by MAPK, the NF-kB or Akt pathways is unclear and requires further experiments. ◊
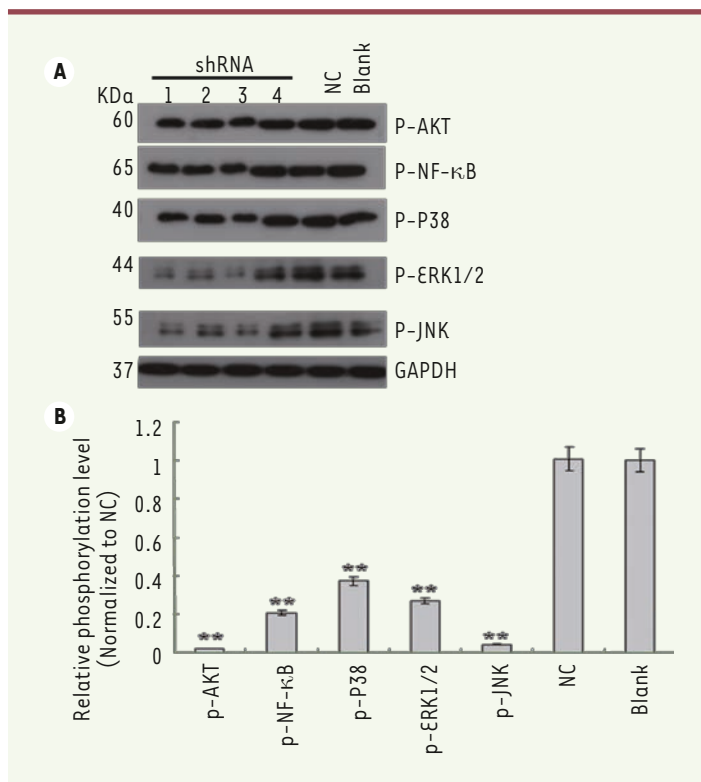
Figure 3. *Filaggrin knock-down in NHEKs affects MAPK signaling pathways.* ***A.*** Expression of p-Akt, p-NF-κB, p-p38, p-ERK and p-JNK detected by Western blotting after shRNA1 transfection into NHEKs. ***B.*** Semi-quantitative analysis of p-Akt, p-NF-κB, p-p38, p-ERK and p-JNK, compared to the control group (NC). n=3, ** $P<0.001$.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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