Dysregulated function of normal human epidermal keratinocytes in the absence of *filaggrin*

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Abstract. The aim of the present study was to investigate the impact of *filaggrin* knockdown on the function of normal human epidermal keratinocytes (NHEKs). *Filaggrin* expression levels in NHEKs were knocked down by lentivirus (LV) encoding small hairpin RNA (shRNA), with control cells infected with nonsense shRNA or not infected. Cell migration and invasion were assayed using Transwell inserts, cell adhesion and proliferation by the Cell Counting kit-8 assay, and apoptosis and cell cycle progression by flow cytometry. shRNA efficiently suppressed expression of filaggrin protein. The LV group had significantly decreased cell migration, adhesion and proliferation, and increased apoptosis compared with the control groups (P=0.027). In addition, the proportion of cells in G_1 and G_2 phases were significantly increased in the LV group compared with control groups (P=0.018). The results of the present study demonstrate that *filaggrin* knockdown inhibits NHEK migration, adhesion and proliferation, promotes apoptosis and disturbs cell cycle progression.

Introduction

The *FLG* gene, which encodes filament aggregating protein (filaggrin), is located on human chromosome 1q21 (1). Filaggrin is a filament-associated protein that binds to keratin fibers in epithelial cells. Filaggrin monomers cluster into profilaggrin, which is processed into filaggrin monomers by proteolysis. Filaggrin is crucial for epidermal homeostasis and contributes to the construction of the lipid envelope, which is critical for skin barrier function (2). It is a critical component of the stratum corneum, which provides primary protection in humans due to its physical strength, hydration status, skin pH and buffering capacity (3).

The importance of filaggrin in the frontline skin barrier (4) is demonstrated by the predisposition of individuals with *filaggrin* mutations to various conditions, including dry skin, ichthyosis and atopic dermatitis (5-7). Thus, it is necessary to fully understand the functions of filaggrin to facilitate the treatment of these diseases. It has been demonstrated that filaggrin expression in keratinocytes results in decreased proliferation, post-G_1 phase arrest and loss of cell-cell adhesion (8). In addition, filaggrin increases the susceptibility of keratinocytes to apoptosis in response to apoptosis-inducing stimuli (9). Furthermore, there is evidence to suggest that filaggrin contributes to nuclear events associated with apoptosis of epidermal keratinocytes (10). However, the effect of *filaggrin* knockdown on the functions of normal human epidermal keratinocytes (NHEKs) remains to be fully elucidated.

In the present study, the effect of *filaggrin* absence on migration, invasion, adhesion, proliferation, apoptosis and cell cycle progression in NHEKs was investigated. The results of the present study may facilitate the determination of the pathogenesis of *filaggrin* mutation-associated disorders.

Materials and methods

Cell culture. NHEKs were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA), and cultured in EpiLife® medium supplemented with growth factors (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured in 10 cm dishes in a 5% CO_2 incubator at 37˚C. The medium was replaced every second day and the cells were split 1:2 every 3 days. For experiments other than cell proliferation and adhesion, cells were cultured with 1.5 mM calcium for 24 h to induce differentiation.

Filaggrin silencing by LV infection. The present study used the following LV-encoding shRNA infection to knockdown *filaggrin*: GTTGGCTCAAGCA TATTATTT (position: nt-274). The negative control (NC) shRNA sequence was CAACAAGATGAAGAGCACC. The complementary DNA of the shRNA was inserted into the LV gene transfer vector and the double stranded shRNA oligo was cloned into pGLV-H1-GFP (Shanghai GenePharma Co., Ltd., Shanghai,

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China) with *Bam*HI and *Eco*RI (Thermo Fisher Scientific, Inc.). The construct was validated by western blotting. The shRNA-infected cells were referred to as the LV group, cells infected with control non-*filaggrin* shRNA as the NC group, and cells without infection as the blank group.

The constructs were diluted 1:4 with EpiLife® medium containing 10% fetal calf serum (FCS; Invitrogen; Thermo Fisher Scientific, Inc.) and 10 mg/ml polybrene® (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), to a final concentration of 5 μg/ml; this was the LV working solution. When the NHEKs reached 90% confluence, the cells were digested using 1 ml 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, and single cell suspensions were prepared. Cells were seeded into 6-well plates ($1x10^6$ cells/well) and incubated in EpiLife® medium containing 10% FCS at 37˚C and 5% CO_2 for 24 h. Following this, the EpiLife® medium was removed and 1 ml LV working solution was added to each experimental well and incubated for 24 h. Cells were observed under a fluorescence microscope (Olympus America, Inc., Melville, NY, USA). The results of the preliminary experiments revealed that LV was stably expressed for four days.

Migration assays. Cell migration was analyzed using Transwell inserts with an 8-μm pore membrane (BD Biosciences, San Jose, CA, USA) as described previously (11,12). The LV-infected cells were grown to sub-confluence (75-80%) and then serum-starved for 24 h. Following detachment with trypsin, the cells were washed with phosphate-buffered saline (PBS) and resuspended in serum-free medium. Subsequently, 100 μl cell suspension ($3x10^5$ cells/ml) was added to the upper chamber. The membranes were coated with 0.01% collagen type I in 0.01 N HCl (Sigma-Aldrich, St. Louis, MO, USA). The lower chamber was filled with 700 μl RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 15% FCS. Following a 48-h incubation in a 5% CO_2 incubator at 37˚C, membranes were removed. Cells remaining on the upper side of the membranes were wiped off using cotton swabs, while cells that had migrated to the lower chamber were fixed with 500 μl methanol for 10 min at -20˚C and stained with 200 μl 0.1% crystal violet for 30 min at 37˚C. Images of five separate fields selected at random (magnification, x100) were captured from each well and the number of migrated cells was counted. The mean number of migrated cells per field was calculated for each experimental condition.

Cell adhesion and proliferation assay. Flat-bottom culture plates (96-well) were coated with 60 μl of Matrigel diluted 1:5 in serum-free EpiLife® medium, incubated in 5% CO_2 at 37˚C for 4 h. NC, blank and LV-treated cells were harvested with 1 ml trypsin-EDTA solution 48 h following LV infection, washed twice with PBS and resuspended in EpiLife® medium. Cells were added to the coated 96-well plates ($5x10^4$ cells/well) in quintuplicate and incubated at 37˚C for 3 h. Subsequently, the 96-well plates were washed twice with PBS to remove unbound cells and 100 μl fresh medium was then added to each well. The remaining adhesive cells in the plate were assessed using a Cell Counting kit (CCK)-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Finally, 96-well plates were examined at 450 nm using a plate reader 2.5 h later. The results were calculated with the following formula: Adhesion rate=[mean optical density (OD) of treated cells]/(mean OD of corresponding control)x100.

In addition, the cell proliferation assay was performed with the CCK-8 (13). Cells were seeded in 96-well plates at ~$5x10^3$ cells/well and cultured in EpiLife® medium at 37˚C. At the indicated time points (0, 24, 48, 72 and 96 h), 10 μl CCK-8 solution was added to each well and incubated for 2.5 h, followed by examination at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA.).

Cell apoptosis assays. A total of 72 h following LV infection, cells were harvested with EDTA free-trypsin for apoptosis analysis or passaged and cultured at a density of $5x10^5$ cells/ml in a 6-well plate for 48 h prior to apoptosis analysis. Analysis was performed using an annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions (14). Briefly, following washing twice with cold PBS and centrifugation at 300 x g for 5 min at 4˚C, the cells were resuspended in 50 μl binding buffer and 5 μl 7-AAD for 15 min at room temperature in the dark. Cells were then incubated with 450 μl binding buffer and 1 μl annexin V-PE for 15 min in the dark and detected immediately on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software version 3.2 (BD Biosciences).

Cell cycle analysis. Following LV infection, cells were incubated at a density of $5x10^5$ cells/ml in a 6-well plate for 48 h. Cells (1-$5x10^6$ cells/ml) were then washed twice with ice-cold PBS, resuspended in 500 μl PBS and fixed with 1.5 ml of precooled 100% ethanol overnight at 4˚C. Following two PBS washes, the fixed cells were centrifuged (300 x g, 5 min, 4˚C) to remove the ethanol. Cells were adjusted to 1-$10x10^5$ cells/ml and incubated with 150 μl RNaseA (250-500 μg/ml) for 30 min at 37˚C, followed by the addition of 100 μl propidium iodide (Sigma-Aldrich) for 30 min at 4˚C in the dark. DNA content was analyzed using a FACSCalibur flow cytometer at an excitation wavelength of 488 nm (15).

Western blotting. Cells were harvested 72 h following LV infection and washed twice with ice-cold PBS. Briefly, the cells were lysed and homogenized with radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) for 30 min on ice. Samples were diluted with 2X SDS-PAGE loading buffer (1:1), followed by thermal denaturation at 100˚C for 5 min. Following cooling, the supernatants were collected by centrifugation at 10,000 x g at 4˚C for 10 min. The protein concentration was quantified by a bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Protein samples (30 mg) were separated using a 10% SDS-PAGE gel (100 mV), and transferred to nitrocellulose membranes. Following blocking with 5% nonfat milk in Tris-buffered saline and Tween 20 (TBST) for 2 h, the membranes were incubated overnight at 4˚C with primary antibodies: Rabbit anti-filaggrin monoclonal antibody (catalog no. PRB-417P; 1:250) purchased from Covance, Inc. (Princeton, NJ, USA) and

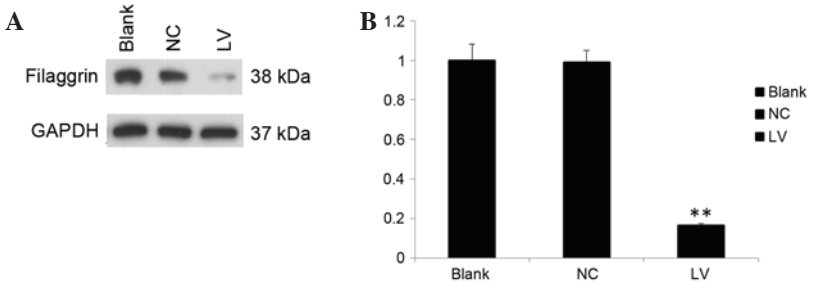

Figure 1. *Filaggrin* knockdown in NHEKs. (A) Western blot analysis of *filaggrin* protein expression levels in NHEKs. Cells were infected with LV encoding shRNA targeting *filaggrin* (LV group), LV encoding nonsense shRNA (NC group) or uninfected (blank group). Filaggrin protein expression levels were determined 72 h following LV infection, with GADPH serving as the internal reference. (B) Quantification of western blot analysis. The expression level of filaggrin protein was significantly inhibited. **P<0.01 vs. NC group. NHEKs, normal human epidermal keratinocytes; LV, lentivirus; NC, negative control.

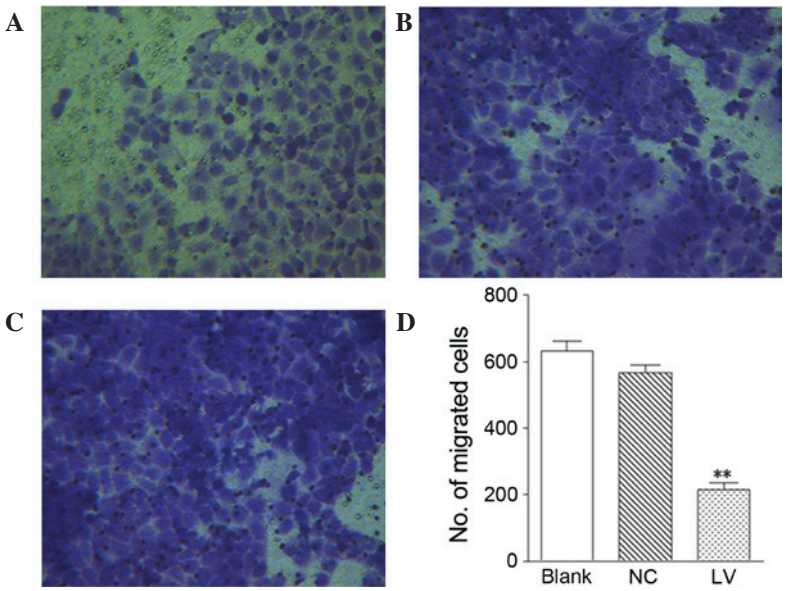

Figure 2. Effect of *filaggrin* knockdown on migration and invasion of NHEKs. Transwell inserts were used to investigate the migration of NHEKs. Following incubation, cells that had migrated to the lower chamber were stained and analyzed. Images of migrated NHEKs in the (A) LV, (B) NC and (C) blank groups are presented. (D) The number of migrated NHEKs was counted in five random fields in the LV, NC and blank groups, and presented as the mean ± standard deviation. The number of migrated NHEKs in the LV group was significantly reduced compared with the NC and blank groups (**P<0.01 vs. the NC group). Magnifications, x100. NHEKs, normal human epidermal keratinocytes; LV, lentivirus; NC, negative control.

rabbit anti-GAPDH antibody (catalog no. sc-25778; 1:3,000) purchased from Santa Cruz Biotechnology, Inc. Membranes were washed with TBST three times and incubated with a goat-rabbit IgG secondary antibody conjugated to horseradish peroxidase (catalog no. sc-2030; 1:1,000) purchased from Santa Cruz Biotechnology, Inc. Reactive bands were detected by enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). Protein expression levels were quantified using Gel-Pro Analyzer software version 3.1 (Media Cybernetics, Inc., Rockville, MD, USA). GAPDH served as the internal reference.

Statistical analysis. Data are presented as the mean ± standard deviation, and all statistical analyses were conducted using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Differences between the experimental groups were analyzed using Student's *t*-test, or one-way analysis of variance followed by the least significant difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

shRNA infection effectively knocks down filaggrin expression levels. The effect of shRNA infection on the protein expression levels of filaggrin was determined by western blotting. As presented in Fig. 1, shRNA infection resulted in a significant decrease in filaggrin protein expression levels at 72 h (P=0.008 vs. NC group). The results of the present study indicated that *filaggrin* was successfully knocked down.

Filaggrin knockdown inhibits cell migration. The impact of *filaggrin* knockdown on cell migration was investigated using Transwell inserts. As presented in (Fig. 2A-D), the LV group (Fig. 2A) had significantly less migrated cells than the NC (Fig. 2B; P=0.0059) and blank groups (Fig. 2C). This observation suggested that a lack of *filaggrin* may markedly inhibit the migration of NHEKs.

Filaggrin knockdown suppresses cell adhesion and proliferation. In addition to cell migration and invasion, the role of

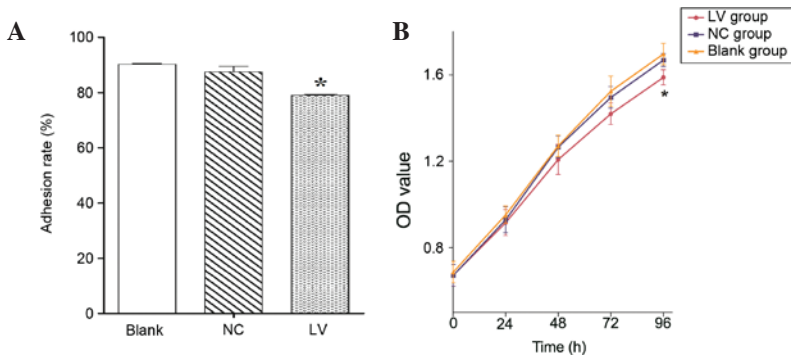

Figure 3. Effect of *filaggrin* knockdown on adhesion and proliferation of NHEKs. NHEK cells were infected with LV encoding *filaggrin* shRNA (LV), nonsense shRNA (NC) or uninfected (blank), following which adhesion and proliferation were analyzed using a Cell Counting kit-8. (A) The LV group exhibited decreased cell adhesion compared with the NC and blank groups. Adhesion rate=(mean OD of treated cells)/(mean OD of corresponding control)x100. (B) The OD values of NHEK cells at 0, 24, 48, 72 and 96 h following infection. *P<0.05 vs. NC group. NHEKs, normal human epidermal keratinocytes; LV, lentivirus; NC, negative control; OD, optical density.

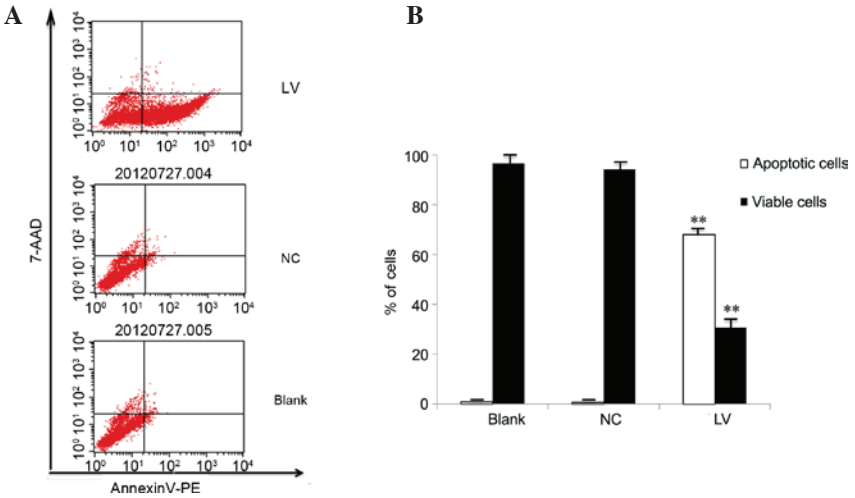

Figure 4. Impact of *filaggrin* knockdown on apoptosis of NHEKs (72 h following LV infection). (A) Apoptosis analysis of LV, NC and blank groups by flow cytometry. Representative plots of annexin V-PE/7-AAD staining are presented. The population staining positive for annexin V and negative for 7-AAD (lower right quadrant) was defined as apoptotic. (B) The statistical results of the proportion of apoptotic and viable (lower left quadrant) cells in LV, NC and Blank groups 72 h following infection. **P<0.01 vs. NC group. NHEKs, normal human epidermal keratinocytes; LV, lentivirus; NC, negative control; 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin.

filaggrin in cell adhesion and proliferation was analyzed. As presented in Fig. 3A, adhesion of the LV group was significantly inhibited compared with the NC (P=0.023) and blank groups. Furthermore, *filaggrin* knockdown had no significant influence on cell proliferation at 72 h; however, a significant decrease was observed at 96 h in the LV group compared with the NC (P=0.034) and blank groups (Fig. 3B).

Filaggrin knockdown induces apoptosis. An annexin V-PE/7-AAD apoptosis detection kit was used to determine the effect of *filaggrin* knockdown on apoptosis. As presented in Fig. 4, the proportion of early apoptotic cells was significantly increased in the LV group compared with the NC and blank groups (68.01 vs. 0.76 and 0.92%, respectively; P=0.0065 vs. NC group), while the proportion of viable cells was significantly decreased (30.60 vs. 94.00 and 96.6%; P=0.0063 vs. NC group), at 72 h following infection. Passaged cells (72 h following infection and 48 h following passage), exhibited a similarly significant increase in the percentage of early apoptotic cells (72.14 vs. 0.56 and 0.82%; P=0.0054 vs. NC group) and a significant decrease in the proportion of viable cells (25.88 vs. 94.22 and 95.23%; P=0.0076 vs. NC group; Fig. 5). Therefore, *filaggrin* knockdown appeared to induce apoptosis of NHEKs.

Filaggrin knockdown alters cell cycle progression. Flow cytometry was performed to evaluate the effect of *filaggrin* knockdown on cell cycle progression in NHEKs. As presented in Fig. 6A-C, the cell cycle distribution pattern of the LV group was distinct to that of the NC and blank groups. The proportion of cells in S phase was significantly reduced (18.19 vs. 25.90 and 26.07%; P=0.034 vs. NC group); however, the proportion of cells in G_1 and G_2 phases was significantly increased in the LV group compared with the NC and Blank groups (71.82 vs. 65.29 and 66.25%; P=0.031 vs. NC group; and 3.47 vs. 7.92 and 6.89%; P=0.0064 vs. NC groups). The increase in cells that had undergone *filaggrin* knockdown in G_1 and G_2 stages suggested that G_1/S transition was inhibited and S/G_2 transition accelerated as a result of *filaggrin* knockdown.

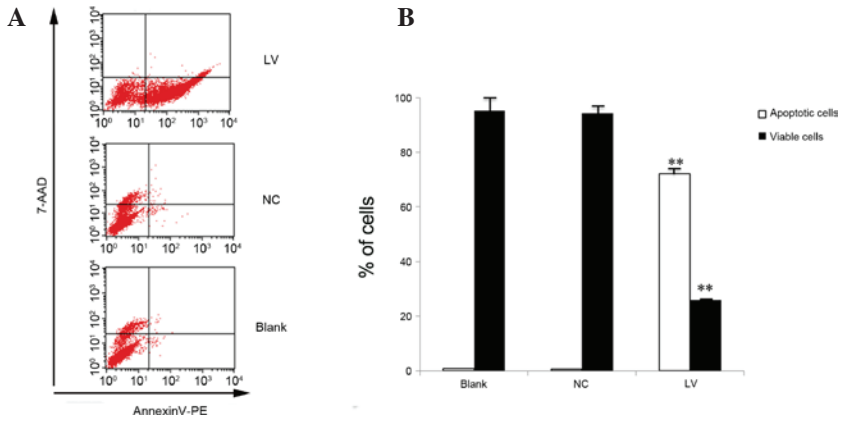

Figure 5. Impact of *filaggrin* knockdown on apoptosis of NHEKs (72 h following infection and 48 h following passage). (A) Apoptosis analysis of LV, NC and blank groups by flow cytometry. Representative plots of annexin V-PE/7-AAD staining are presented. The population staining positive for annexin V and negative for 7-AAD (lower right quadrant) was defined as apoptotic. (B) Quantification of the proportion of apoptotic and viable (lower left quadrant) cells in the LV, NC and blank groups 72 h following infection and 48 h following passage. **P<0.01 vs. NC group. NHEKs, normal human epidermal keratinocytes; LV, lentivirus; NC negative control; 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin.

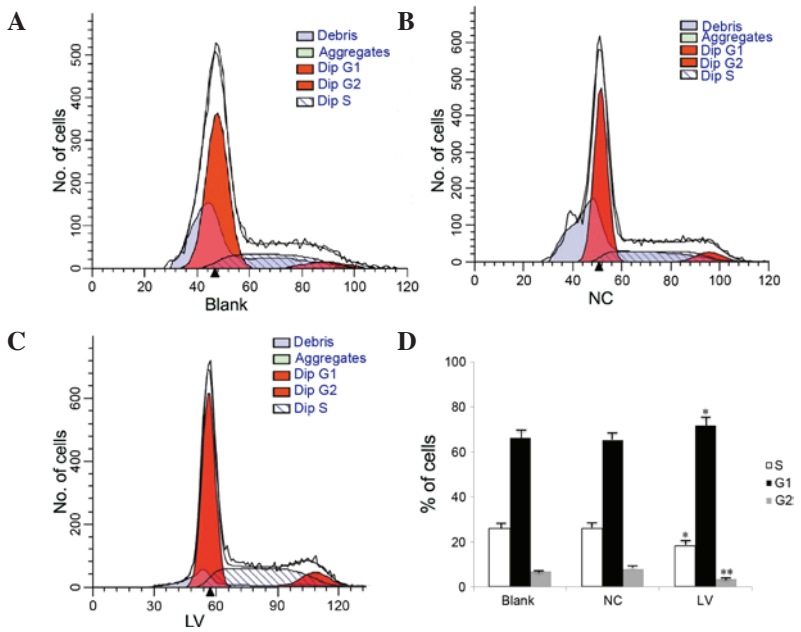

Figure 6. Effect of *filaggrin* knockdown on the cell cycle of NHEKs (72 h following LV infection). Cell cycle analysis of NHEKs in (A) LV, (B) NC and (C) blank groups by flow cytometry. (D) Quantification of the proportion of cells in S (Dip S), G_1 (Dip G1) and G_2 (Dip G2) phases in the LV, CN and blank groups. *P<0.05 and **P<0.01 vs. NC group. NHEKs, normal human epidermal keratinocytes; LV, lentivirus; NC, negative control.

Discussion

Filaggrin mutations may contribute to various diseases, including dry skin, ichthyosis vulgaris, atopic eczema and atopic dermatitis (16). Filaggrin is crucial for epidermal homeostasis and differentiation, and skin barrier function (17). The present study aimed to investigate the effects of *filaggrin* knockdown on various functions of NHEKs, including cell migration, invasion, adhesion, proliferation, apoptosis and cell cycle progression. *Filaggrin* was successfully knocked down in NHEKs by infection with LV encoding shRNA of *filaggrin*, resulting in inhibition of cell migration, adhesion and proliferation, promotion of apoptosis, and disturbance of cell cycle progression.

Cell migration is a highly integrated multistep process, which contributes to tissue formation, regeneration and remodeling, wound healing, and the immune response (18). Tissue repair, regeneration and remodeling require active cell motility, which is also reliant on cell adhesion (19). In addition, cell adhesion is involved in the maintenance of multicellular structures, signal transduction and cancer metastasis (20). Cell migration and adhesion are closely associated with terminal differentiation and epidermal homeostasis (21,22). Our previous study demonstrated that *filaggrin* knockdown inhibits expression of epidermal differentiation-associated proteins (23). Consistent with these findings, the present study revealed suppression of NHEK migration and adhesion as a result of *filaggrin* knockdown. Therefore, the absence of *filaggrin* may prohibit epidermal differentiation by suppressing the migration and adhesion of NHEKs.

Furthermore, *Filaggrin* knockdown inhibited cell proliferation and promoted apoptosis. Cell proliferation and apoptosis are involved in epidermal homeostasis and skin wound repair (21,24). These observations indicated that the absence of *filaggrin* may deregulate epidermal homeostasis and delay wound healing by inhibiting NHEK migration, adhesion and proliferation, and promoting NHEK apoptosis.

Cell cycle progression in the present study was also altered by *filaggrin* knockdown, as indicated by the increased proportions of cells in G_1 and G_2 phases, and the reduced proportions of cells in S phase. Therefore, *filaggrin* absence slowed down G_1/S transition and accelerated S/G_2 transition, which may provide an explanation for the inhibition of proliferation by *filaggrin* knockdown. These results suggest that *filaggrin* is involved in the regulation of cell cycle progression; however, further studies are required to validate this effect.

Evidence suggests that the MAPK and phosphoinositide 3-kinase/Akt signaling pathways are involved in regulating keratinocyte differentiation, proliferation and apoptosis (25-28). Downregulation of Akt has been reported to promote apoptosis, and inhibit cell migration and proliferation (29). Activation of Akt reverses cell cycle arrest in G_1 and G_2 phases in response to DNA injury (30). Furthermore, the effect of Akt on cellular survival and metabolism is mediated by binding to downstream NF-κB (31). *Filaggrin* absence may inhibit the differentiation of NHEKs by suppressing phosphorylation of P38, ERK1/2, JNK, Akt and NF-κB. Therefore, *filaggrin* knockdown may inhibit cell migration, adhesion and proliferation, promote cell apoptosis and disturb cell cycle progression via suppression of these signaling pathways. However, further experiments are required to confirm this hypothesis.

In conclusion, the results of the present study demonstrate that *filaggrin* knockdown inhibits NHEK migration, adhesion and proliferation, promotes apoptosis and disturbs cell cycle progression. These findings contribute to the understanding of the role of *filaggrin* in epidermal keratinocytes, and may facilitate the determination of the pathogenesis of *filaggrin* mutation-associated disorders.

Acknowledgements

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