Proteomic analysis of filaggrin deficiency identifies molecular signatures characteristic of atopic eczema

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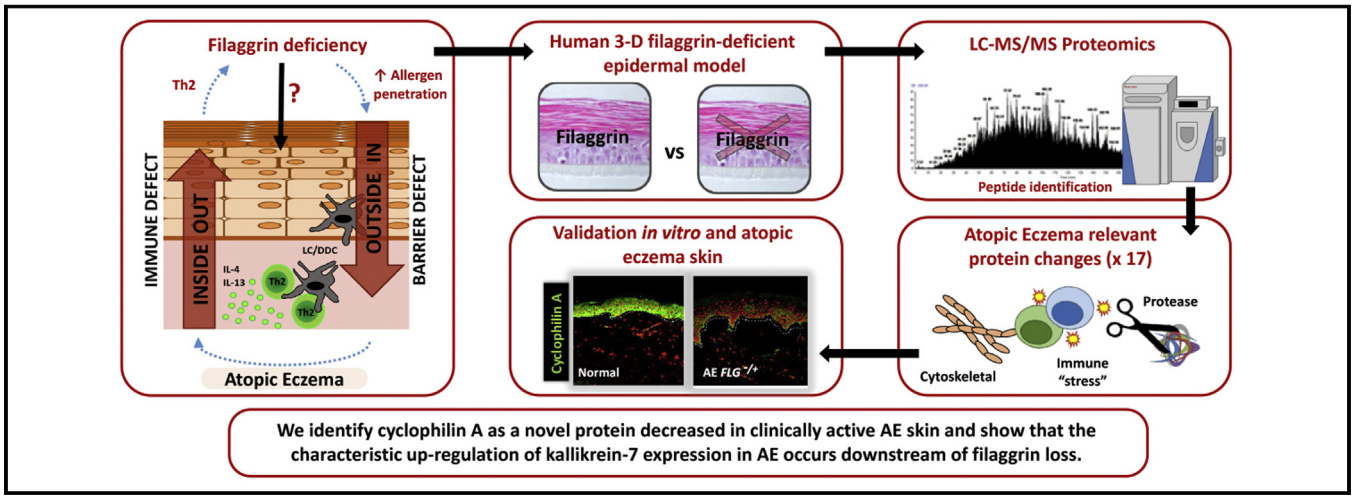
GRAPHICAL ABSTRACT



Background: Atopic eczema (AE) is characterized by skin barrier and immune dysfunction. Null mutations in filaggrin (FLG), a key epidermal barrier protein, strongly predispose to AE; however, the precise role of FLG deficiency in AE pathogenesis remains incompletely understood.

Objectives: We sought to identify global proteomic changes downstream of FLG deficiency in human epidermal living skin–equivalent (LSE) models and validate findings in skin of patients with AE.

Methods: Differentially expressed proteins from paired control (nontargeting control short hairpin RNA [shNT]) and FLG knockdown (FLG knockdown short hairpin RNA [shFLG]) LSEs were identified by means of proteomic analysis (liquid chromatography–mass spectrometry) and Ingenuity Pathway Analysis. Expression of key targets was validated in independent LSE samples (quantitative RT-PCR and Western blotting) and in normal and AE skin biopsy specimens (immunofluorescence).

Results: Proteomic analysis identified 17 ($P \leq .05$) differentially expressed proteins after FLG knockdown, including kallikrein-7 (KLK7; 2.2-fold), cyclophilin A (PPIA; 0.9-fold), and cofilin-1 (CFL1, 1.3-fold). Differential protein expression was confirmed in shNT/shFLG LSEs; however, only KLK7 was transcriptionally dysregulated. Molecular pathways overrepresented after FLG knockdown included inflammation, protease activity, cell structure, and stress. Furthermore, KLK7 (1.8-fold) and PPIA (0.65-fold) proteins were differentially expressed in

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lesional biopsy specimens from patients with AE relative to normal skin.

Conclusions: For the first time, we show that loss of FLG in the absence of inflammation is sufficient to alter the expression level of proteins relevant to the pathogenesis of AE. These include proteins regulating inflammatory, proteolytic, and cytoskeletal functions. We identify PPIA as a novel protein with levels that are decreased in clinically active AE skin and show that the characteristic upregulation of KLK7 expression in patients with AE occurs downstream of FLG loss. Importantly, we highlight disconnect between the epidermal proteome and transcriptome, emphasizing the utility of global proteomic studies. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Atopic eczema, dermatitis, skin, proteomic, filaggrin, kallikrein-7, cyclophilin A*

Abbreviations used

AE:	Atopic eczema
ANXA3:	Annexin A3
CFL1:	Cofilin-1
CTSV:	Cathepsin V
eIF:	Eukaryotic initiation factor
FLG:	Filaggrin
IPA:	Ingenuity Pathway Analysis
KLK7:	Kallikrein-7
LSE:	Living skin equivalent
mTOR:	Mammalian target of rapamycin
PPIA:	Cyclophilin A
RT-qPCR:	Quantitative RT-PCR
shNT:	Nontargeting control short hairpin RNA
shFLG:	Filaggrin knockdown short hairpin RNA
TXN:	Thioredoxin
VIM:	Vimentin

Filaggrin (FLG) is a major constituent of the epidermal barrier, contributing to its structure, function, and hydration.[1] Both single- and double-allele loss-of-function mutations in *FLG* strongly predispose to the development of atopic eczema (AE) and secondary atopic conditions, including asthma and allergic rhinitis.[2-4] To date, more than 40 different population-specific *FLG* mutations have been identified, each resulting in a truncated profilaggrin gene product, which is not processed into functional FLG monomers.[3] Up to 50% of cases of moderate-to-severe AE in northern Europe can be attributed, at least in part, to an *FLG*-null mutation,[5,6] representing the strongest and most consistent genetic risk factors identified for AE to date (overall odds ratio, 3.12-4.78).[6,7] Furthermore, *FLG* is polymorphic, with common allelic variants encoding a profilaggrin molecule composed of 10, 11, or 12 FLG monomeric repeats. Each additional FLG repeat confers a reduced risk of AE by a factor of 0.88, suggesting that even small increases in *FLG* expression might be therapeutically beneficial.[8]

Both the flaky tail mouse (which carries a natural mutation virtually ablating all FLG expression[9]) and FLG knockout mice exhibit enhanced percutaneous antigen transfer and allergen/irritant-induced AE-like inflammatory responses.[10,11] These models support a primary role for FLG and highlight the importance of a functional cutaneous barrier in AE disease pathogenesis.

Recent cohort studies have examined global gene expression changes in patients with AE stratified by FLG genotype.[12,13] Although interesting, their interpretation with regard to the direct role of FLG in AE pathogenesis is confounded by the complex interplay between the epidermal barrier, immune system, and environment.[14] For example, loss of FLG is also observed in patients with *FLG* wild-type AE, likely because of its extrinsic downregulation by the atopic T_H2-polarized microenvironment.[15] Moreover, murine models are additionally limited both by secondary mutations, notably null mutations in *TMEM79/Matt* (Mattrin) in the flaky tail mouse,[16] and interspecies differences. The human and murine *FLG* sequences lack homology,[17] and murine models do not display the heterozygote phenotype typical of most patients with AE.[10,11] Consequently, the precise molecular changes occurring directly as a result of FLG loss are relatively unknown.

A useful alternative tool for the study of epidermal biology is the 3-dimensional living skin–equivalent (LSE) model derived exclusively from primary human keratinocytes. Although by definition these simplified models do not recapitulate the complexity of human AE skin, they enable the study of epidermal biology in the absence of confounding inflammatory cells.[18] Furthermore, they enable the effect of *FLG* gene silencing to be analyzed in a pairwise manner on a homogeneous genetic background.

Previous LSE studies have largely focused on understanding the ultrastructural and functional consequence of FLG deficiency.[19-21] In many regards these mirror changes observed in AE skin, supporting their utility as a disease model. However, to the best of our knowledge, no systematic transcriptomic or proteomic analysis has been performed after *FLG* knockdown in human epidermis. The aim of this study was to use FLG knockdown LSE models to investigate the global molecular consequences resulting directly from *FLG* deficiency. A proteomics-based approach was used because protein changes largely represent the functional end point of cell signaling, and recent studies have identified an important disconnect between the transcriptome and proteome, suggesting that significant posttranscriptional regulation occurs.[22]

Notably, for the first time, we have identified 17 proteins that are significantly differentially expressed after FLG knockdown in LSE cultures. Bioinformatic analysis was used to categorize and align these to putative regulatory networks and showed that loss of FLG alone is sufficient to induce protein changes relevant to the pathogenesis of AE. Specifically, pathways relating to protease activity, inflammation, cell structure, and stress were overrepresented after FLG knockdown. The expression profile of key targets, namely the AE-relevant protease kallikrein-7 (KLK7), the novel AE immune modulator cyclophilin A (PPIA), and the actin-binding protein cofilin-1 (CFL1), were replicated first in independent FLG knockdown LSEs and then further characterized in skin biopsy specimens from patients with AE.

METHODS

Primary keratinocyte culture

Normal human epidermal keratinocytes extracted from surplus foreskin tissue obtained after informed biobank consent were cultured in low-calcium (0.06 mmol/L) EpiLife supplemented with 1% human keratinocyte growth supplement (Life Technologies, Paisley, United Kingdom), as previously described.[23] All donor subjects had no history of AE.