Current Perspectives Series: Filaggrin in Atopic Dermatitis

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ABBREVIATIONS: CE: cornified cell envelope; EDC: epidermal differentiation complex; *FLG*: filaggrin; HDM: house-dust mite; IV: ichthyosis vulgaris; *KLK7*: kallikrein 7; LEPs: late envelope proteins, LEKTI: lymphoepithelial Kazal-type trypsin inhibitor; NMF: natural moisturizing factor; PCA: pyrolidone carboxylic acid; SC: stratum corneum; *SPINK5*: serine protease inhibitor kazal type 5; SSCE: stratum corneum chemotryptic enzyme; TSLP: thymic stromal lymphopoietin; UCA: urocanic acid.

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Abstract

The recent identification of loss-of-function mutations in the structural protein filaggrin as a widely replicated major risk factor for eczema sheds new light on disease mechanisms in eczema, a disease which had heretofore largely been considered to have a primarily immunological aetiopathogenesis. The filaggrin mutation findings are consistent with a recently proposed unifying hypothesis that offers a mechanistic understanding of eczema pathogenesis synthesizing a heritable epithelial barrier defect and resultant diminished epidermal defense mechanisms to allergens and microbes, followed by polarized T_H2 lymphocyte responses with resultant chronic inflammation, including auto-immune mechanisms. Although compelling evidence from genetic studies on filaggrin implicates perturbed barrier function as a key player in the pathogenesis of eczema in many patients, much is still unknown about the sequence of biological, physicochemical and aberrant regulatory events that constitute the transition from an inherited barrier defect to clinical manifestations of inflammatory eczematous lesions and susceptibility to related atopic disorders. The exact contribution of filaggrin to the wider atopic story, factors modifying filaggrin expression and the role of other barrier proteins remain to be delineated. In this review we highlight recent advances in our understanding of the *FLG* genetics in the etiology of eczema and related complex diseases.

The epidermal barrier: structure and function

The epidermis provides an essential attribute of adaptation to terrestrial life, namely an occlusive interface barrier, restricting both water loss from the body and ingress of pathogens. This barrier is formed after a complex, integrated and exquisitely regulated series of biochemical events cumulating in a program of cell death by terminally differentiating keratinocytes [1]. In order to achieve and maintain this barrier, epithelial keratinocytes replace their plasma membrane with a tough, insoluble macromolecular layer, called the cornified envelope (CE).

Initial steps in the formation of the cornified envelope result in the sequential expression of several major protein products, but only certain proteins from a choice of more than 20 are used in the final stages of CE reinforcement, in order to meet site-specific requirements, of which filaggrin (*FLG*) is one of the final proteins to be incorporated [1]. These structural proteins are extensively cross-linked by transglutaminases and act as a scaffold for the attachment of a layer of lipids covalently bound to the extracellular surface, forming an outer lipid envelope. In response to deficiency, injury or other environmental triggers, proteins forming the CE may be upregulated in an effort to compensate and maintain an effective barrier [1]. Cell differentiation, death and desquamation occur sequentially, with recent convergent approaches affording insight into the molecular mechanisms and diseases associated with defects in the pathways of cornification.

Many of the key proteins involved in cornification are encoded for in a gene-dense locus on chromosome 1q21, termed the epidermal differentiation complex (EDC) [2]. The EDC spans an area of 1.62 megabases, containing more than 70 genes expressed during the late stages of terminal differentiation, and genome wide screens have shown significant linkage co-localization with psoriasis, autoimmune diseases and eczema, heightening interest in this locus [3]. Many EDC proteins share significant sequence similarities, and phylogenic data suggests that these proteins derived from a common ancestor, evolving to meet tissue-specific demands. These genes cluster within the EDC according to expression pattern, and are in tight linkage disequilibrium, suggesting that they may also be co-regulated. Genes found within this locus encode for proteins such as loricrin, involucrin, small proline-rich proteins (SPRs), late envelope proteins (LEPs), and the S100 calcium-binding proteins, of which *FLG* is a key member. There is considerable evidence for redundancy mechanisms in CE assembly, in that the absence of one CE reinforcement protein can be compensated by increased expression of others in experimental animal models [4]. This is exemplified by the *loricrin*$^{-/-}$ mouse, which displays a mild epidermal erythema at birth that normalizes within days, in spite of the fact that loricrin typically comprises 70-85% of the protein content of CE. This phenotype is associated with the compensatory upregulation of the EDC structural proteins Sprr2D, Sprr2H and repetin. Targeted ablation of the murine involucrin gene, a near-ubiquitous component of CE, similarly lacks a discernable phenotype. The recent

development of a composite triple-knock out mouse deficient in involucrin, envoplakin and periplakin demonstrates delayed barrier formation during embryogenic development, defects in the assembled CE, and excessive accumulation of cornified layers throughout postnatal life, suggesting that these initiator CE proteins are critical for barrier acquisition [4]. Strikingly, reduced epidermal protease activity, as opposed to upregulation of structural proteins, rescues the phenotype from lethality, with a marked increase of the protease inhibitor *serpina1b*, resulting in a compensatory reduction in desquamation, with secondary downstream defects in defective *FLG* processing [4]. For a more detailed consideration of the epidermal barrier in atopic dermatitis, please see an earlier article in this series by Elias et al[5].

Filaggrin expression and function

The giant inactive precursor, profilaggrin is a large, complex, highly phosphorylated polypeptide that is the main constituent of the keratohyalin F granules that are visible in the granular cell layer of the epidermis (Figure 1A). During formation of the cornified cell envelope, profilaggrin is dephosphorylated and proteolytically cleaved by serine proteases including CAP1/Prss and matriptase/MT-SP1 to release multiple copies of the functional filaggrin repeat peptide units. Control of protease activity is balanced by a series of inhibitors, which are abundant and pivotal in epithelial differentiation, the most characterized of which is lymphoepithelial Kazal-type trypsin inhibitor (LEKTI), the polyvalent protein product encoded

by *SPINK5*. Following cleavage, liberated filaggrin binds to and collapses the keratin cytoskeleton, resulting in a flattened squame aligned parallel to the outer surface of the epidermis. The cleaved N-terminal S100-like calcium binding domain of profilaggrin enters the nucleus, where it is postulated to have an additional role in regulating terminal differentiation. Subsequently, within the stratum corneum (SC) itself, the filaggrin peptide is progressively degraded by post-translational modification enzymes (including peptidylarginine deiminase (PAD 1 and 3) isoforms) into a pool of hydrophilic amino acids including urocanic acid (UCA), pyrrolidone carboxylic acid (PCA) and alanine. This combined pool of amino acids, their metabolites and various ions make up what is known as the Natural Moisturising Factor (NMF) [6](Figure 1C). NMF is highly hygroscopic and plays a central role in maintaining hydration of the SC. NMF may additionally play a critical role in the maintenance of the pH of the skin, regulating key biochemical events, including protease activity, barrier permeability and cutaneous antimicrobial defense; functions that are fundamentally linked and co-regulated. The importance of filaggrin-derived breakdown products and the profound impact on barrier function in their absence is underscored by the remarkably short half-life of filaggrin, which exists for only 6 hours before full proteolysis. Expression of *FLG* and subsequent activation of hydrolysis of filaggrin peptides into NMF are additionally determined by the properties of the microenvironment, including local pH, external humidity and transepidermal water loss [6].

Filaggrin mutations confer strong genetic susceptibility to eczema

The discovery of the association of *FLG* mutations with atopic diseases followed insights into a common disorder of keratinization, ichthyosis vulgaris (IV). IV is the most common of the ichthyotic disorders, estimated to affect 1 in 250 individuals, and is characterized by generalized fine white scale, palmoplantar hyperlinearity and keratosis pilaris. For over 20 years much indirect evidence pointed towards mutations in *FLG* as causative, however many confounders delayed confirmation of this association. These included inconsistencies in the reported inheritance pattern, with apparent dominant and recessive inheritance, erroneously reported linkage, and a repetitive gene sequence limiting amplification. Two loss-of-functions *FLG* mutations (R501X and 2282del4) were ultimately detected using long-range sequencing and multiple alignment techniques, revealing a semidominant pattern of inheritance, with incomplete penetrance [7]. The number of mutations identified has increased dramatically in the past 2 years, each predicting nonsense or out-of frame deletion/insertion mutations, with population-specific patterns emerging worldwide.

To date the number of *FLG* mutations identified in European populations is 20, of which 6 are prevalent and 14 are of low frequency. In Asian populations an additional 17 mutations, of which 8 are prevalent and 9 are of

low frequency have been identified (Figure 2A). Of note, more distal mutations allow limited expression of profilaggrin, but no production of functional filaggrin subunits, implying a critical role of the C-terminus for *FLG* processing, there is also some early evidence of a trend towards reduced penetrance of more distal mutations [8]. The combined allele frequency of the initial mutations translates into a carrier frequency of almost 10% in individuals of European ancestry [8]. This unexpected finding combined with the known clinical association of IV with eczema, and decreased expression of *FLG* in eczema pointed to a possible association in the pathogenesis of eczema. This association has now been unambiguously established in a series of replication studies, making this the one of the most robust gene associations so far identified in complex trait genetics [8, 9 10 11-13], reviewed in [14, 15]. Overall between 18 and 48% of all eczema collections carry *FLG* null alleles [14]. The relatively high allele frequency of several haplotypically independent null alleles in the population is intriguing and suggests that these have not arisen by genetic drift alone but may be as a result of balanced selection due to an as yet unclear evolutionary heterozygote advantage [16].

The *FLG* mutation findings were corroborated in two recent large population-based studies on more than 6700 English children [17] and 3000 German children, in whom the two common *FLG* mutations R501X and 2282del4 and three rare variants were analyzed [18]. In the German study, *FLG* variants increased the risk for eczema three-fold (OR 3.12, 95% CI=2.33-4.17, p=2.5 x10-

206 [14]) with a population attributable risk of 13.5%. Importantly, these mutations
207 are highly associated with allergen sensitization and the subsequent
208 development of asthma associated with eczema, an association that has been
209 consistently reported [14]. At a population level *FLG* mutations appear to confer
210 an overall risk of asthma of approximately 1.8, but only in the context of prior
211 eczema [19]. As *FLG* is not expressed in bronchial mucosa, transcutaneous
212 sensitization is one suggested mechanistic possibility for filaggrin to confer
213 asthma risk [9, 20].
214

215 ## Filaggrin: epistatic effects?

216 Other genetic associations within pathways that modulate filaggrin have been
217 reported including common maternally derived polymorphisms in the serine
218 protease inhibitor *SPINK5* (particularly Glu420Lys), that have been shown to
219 modify the risk of developing eczema, asthma, and IgE, suggesting that this
220 pathway may lie in altered expression of environmental proteases.
221 Pathological loss-of-function mutations in *SPINK5*, as found in Netherton
222 syndrome, are associated with a profound barrier defect and severe atopic
223 diathesis, resulting in unchecked proteolysis by processing enzymes such as
224 matriptase and other serine proteases of an extracellular desmosomal
225 component (corneodesin) and lipid processing enzymes [21]. Gain of function
226 polymorphisms in the *kallikrein 7* gene (*KLK7*) encoding the protease stratum
227 corneum chemotrypic enzyme (SSCE), have been additionally reported to
228 adversely affect barrier function, and are postulated to affect the proteolytic

processing of profilaggrin, and is potentially regulated by LEKTI. Recently we studied these reported mutations in several large patient collections involving more than 2500 patients and 10 000 controls. We were able to confirm a role for maternally inherited *SPINK5* mutations in a German family cohort, but could not replicate the *KLK7* findings. Neither *KLK7* nor *SPINK5* had any epistatic effects with *FLG* null alleles [22].

Filaggrin and eczema pathogenesis: mechanisms and speculations

While the very strong genetic association of *FLG* mutations with eczema is now clear, the mechanistic pathways from inherited filaggrin haploinsufficiency to the typical inflammatory lesions of eczema requires further elucidation. Filaggrin deficiency leads to reduced NMF [23], which is likely a contributor to the xerotic phenotype seen in many patients with eczema. The initiation of the typical inflammatory response is of great interest and with this in mind should be remembered that around 40% of all carriers of *FLG* null alleles never develop any signs of eczema [17]. The environmental and genetic modifiers (discussed above) of this risk are currently unclear, although recent evidence also indicates that filaggrin skin expression could be modulated by the atopic inflammatory response mediated by cytokines IL-4 and IL-13 [24], thus providing a link between this structural molecule and the inflammatory response in eczema.

Other currently speculative mechanisms include the possibility that *FLG* haploinsufficiency may critically modify pH-related altered commensal bacteria expression, thus manipulating host immunity. Altered host immunity to bacterial infections is a notable feature of atopic dermatitis [25]. Growth of *S aureus* is facilitated by increased pH, which colonizes the skin of over 90% of eczema patients. Exposure of a naive immune system to *S. aureus* superantigens may trigger and establish a permanent T_H2 immune response, through activation and amplification of innate immune responses. The neutralizing acid SC pH has also been shown to independently facilitate excessive protease activity, and reduce the activity of key lipid-processing enzymes resulting in the formation of defective lamellar membranes, and a disrupted permeability barrier.

Conclusion

FLG mutations are the strongest and most widely replicated genetic risks for eczema identified to date. They have a clear permissive effect in the early inflammatory effects that characterize eczema, and affect both priming of disease and chronicity. The identification of these mutations has enlivened the field of eczema genetics. Their identification raises the potential for targeted intervention and therapy and may lead to a consideration of a new molecular classification of eczema. The environmental and genetic interactions with *FLG* null alleles that contribute to the pathogenesis of this

distressing, fascinating and complex disease will be of great interest in the next several years.

Figure 1: Filaggrin expression and putative functions in the skin barrier

Schematic summarizing filaggrin expression pattern and putative functions. (A). The precursor pro-protein profilaggrin is strongly expressed within keratohyalin granules, tightly limited to and accounting for the typical appearance of the granular layer. The stratum corneum stains strongly positive for filaggrin. (B). Filaggrin has several proposed site specific functions under the influence of the epidermal terminal differentiation program through the outer granular layer (cleavage of pro-filaggrin to filaggrin), lipid bilayer of the inner stratum corneum (filament compaction, contribution to barrier integrity) and during desquamation of the outer stratum corneum (production of amino acid degradation products that contribute to the hydration of these outer layers and likely contribute to the 'acid mantle') . (C) Summary of current knowledge of molecular control of filaggrin homeostasis. Profilaggrin is dephosphorylated in conditions of increasing calcium concentration and is then proteolytically cleaved by the proteases matripase (inhibited by the protease inhibitor LETKI) and CAP1/Prss. Post-proteolysis, the filaggrin B domain locates to the nucleus as part of the terminal differentiation process. Free filaggrin protein is cross-linked to keratin filaments by transglutaminases and subsequently deiminated by peptidylarginine deiminases (PADs) 1 and 3. Further post-translational modification is undertaken by caspase 14 to produce the free amino acid hygroscopic degradation products urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA) {collectively known as natural moisturizing factor; (NMF)} which contributes to stratum corneum hydration.

Figure 2: Protein organization and location of mutations

FLG is composed of a large transcript encoded by three exons, of which the 3rd exon encodes the *FLG* protein repeats. The bulk of *FLG* protein sequences consist of a tandem array of repeating units of 35 kDa, separated by a 7-10 amino acid linker peptide. There are 10 highly homologous *FLG* polypeptide units; with a variable number of *FLG*-repeat units, consisting of 10, 11 or 12 units. The locations of 37 known mutations are shown. Reported mutations resulting in functional null-alleles are numbered; positional locations of as yet unreported mutations are demonstrated by unmarked arrows. Prevalent mutations are indicated by red font, family-specific mutations are in black (A). Recurrent mutations can occur on the background of a 10, 11 or 12 repeat allele (B).

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