Knockdown of Filaggrin Impairs Diffusion Barrier Function and Increases UV Sensitivity in a Human Skin Model

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Loss-of-function mutations in the filaggrin gene are associated with ichthyosis vulgaris and atopic dermatitis. To investigate the impact of filaggrin deficiency on the skin barrier, filaggrin expression was knocked down by small interfering RNA (siRNA) technology in an organotypic skin model *in vitro*. Three different siRNAs each efficiently suppressed the expression of profilaggrin and the formation of mature filaggrin. Electron microscopy revealed that keratohyalin granules were reduced in number and size and lamellar body formation was disturbed. Expression of keratinocyte differentiation markers and the composition of lipids appeared normal in filaggrin-deficient models. The absence of filaggrin did not render keratins 1, 2, and 10 more susceptible to extraction by urea, arguing against a defect in aggregation. Despite grossly normal stratum corneum morphology, filaggrin-deficient skin models showed a disturbed diffusion barrier function in a dye penetration assay. Moreover, lack of filaggrin led to a reduction in the concentration of urocanic acid, and sensitized the organotypic skin to UVB-induced apoptosis. This study thus demonstrates that knockdown of filaggrin expression in an organotypic skin model reproduces epidermal alterations caused by filaggrin mutations *in vivo*. In addition, our results challenge the role of filaggrin in intermediate filament aggregation and establish a link between filaggrin and endogenous UVB protection.

Journal of Investigative Dermatology (2010) **130,** 2286–2294; doi:10.1038/jid.2010.115; published online 6 May 2010

INTRODUCTION

Epidermal keratinocytes (KCs) undergo distinct morphological and biochemical changes as they move from the basal layer to the spinous and granular layers, eventually forming the stratum corneum. Although keratins 5 and 14 are the main keratins in the basal layer, suprabasal KCs express keratins 1, 2, and 10. In addition, several differentiation-associated proteins uniquely found in the epidermis, such as involucrin, loricrin, and filaggrin, are expressed in the terminally differentiated layers of the epidermis (Watt, 1983; Mehrel *et al.*, 1990; Candi *et al.*, 2005; Denecker *et al.*, 2008). Human profilaggrin is synthesized as a large highly phosphorylated precursor protein of around 500 kDa, containing a calcium binding N terminus, followed by a hydrophilic domain, a truncated filaggrin repeat, 10–12 complete filaggrin repeats, another truncated repeat, and a tail peptide (Gan *et al.*, 1990; Presland *et al.*, 1992; Presland and Dale, 2000; McGrath and Uitto, 2008). During the last steps of terminal KC differentiation, profilaggrin is dephosphorylated, and proteolytically processed into the N-terminal domain and filaggrin monomers (Presland *et al.*, 1997; Presland and Dale, 2000). The N terminus consists of an A domain that contains two Ca^{2+}-binding S100-like EF-hands, and a cationic B domain that contains a functional nuclear localization sequence. It was shown that the filaggrin N terminus is able to translocate into the nucleus and may therefore have a crucial role in nuclear breakdown or other nuclear events of epidermal KC terminal differentiation (Ishida-Yamamoto *et al.*, 1998; Pearton *et al.*, 2002). However, the exact function of the filaggrin N terminus is still unknown. Studies involving keratins and filaggrin purified from skin suggested that filaggrin can interact with different types of intermediate filaments, thereby leading to their aggregation into macrofibrils (Dale *et al.*, 1978). In addition, transient overexpression of human filaggrin *in vitro* results in the collapse of the intermediate filament network (Dale *et al.*, 1997; Presland *et al.*, 2001). In the stratum

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Abbreviations: KC, keratinocyte; siRNA, small interfering RNA; UCA, urocanic acid

Received 12 November 2009; revised 2 March 2010; accepted 17 March 2010; published online 6 May 2010

corneum, filaggrin is degraded into amino acids and other components of the natural moisturizing factors and thereby contributes to the retention of water within corneocytes, this being essential for the osmolarity, flexibility, and barrier function of the stratum corneum (Scott *et al.*, 1982; Scott and Harding, 1986; McGrath and Uitto, 2008).

Clinically, loss-of-function mutations in the filaggrin gene are associated with ichthyosis vulgaris and atopic dermatitis. R501X and 2282del4 are the two most common mutations resulting in a lack of functional filaggrin in populations of European ancestry (Palmer *et al.*, 2006; Smith *et al.*, 2006; McGrath and Uitto, 2008). Three other common mutations and several rare or family-specific mutations are also reported in European ancestral groups (Sandilands *et al.*, 2007, 2009). As filaggrin knockout mice are not available, recent loss-of-function filaggrin studies were performed using the flaky tail (*ft*) mouse as a model system. This spontaneous recessive mouse mutant arose in 1958 on the background of an existing recessive hair phenotype and is characterized by dry and flaky skin and neonatal abnormalities in the tail and paws (Presland *et al.*, 2000). It was shown that these mice express a truncated profilaggrin that cannot be further processed into filaggrin monomers (Presland *et al.*, 2000). The epidermal barrier function is impaired and percutaneous allergen priming is increased in the *ft* mouse, suggesting a similar dysfunction as a cause of skin irritation in human atopic dermatitis (Fallon *et al.*, 2009; Scharschmidt *et al.*, 2009). However, considering the species-specific properties of filaggrin (Harding and Scott, 1983), caution should be taken when interpreting and comparing the function of filaggrin in animal models with that of human filaggrin.

Recently, we have reported the combination of RNA interference technology and human organotypic skin cultures as an efficient method to study gene functions in epidermal development (Mildner *et al.*, 2006). In this study, we knocked down filaggrin in this organotypic skin model to address the questions: (1) whether and how the absence of filaggrin influences the maturation of the epidermal barrier; (2) whether filaggrin deficiency would influence the lipid composition in this model; (3) whether filaggrin is necessary for keratin aggregation in this model; (4) whether Lucifer yellow permeability was compromised in the filaggrin-deficient epidermis; and (5) whether the absence of filaggrin affects the UV sensitivity of KCs in the epidermis.

RESULTS

Filaggrin deficiency in human organotypic epidermis leads to loss of keratohyalin granules and impaired lamellar body formation

Filaggrin expression was knocked down by RNA interference in normal human epidermal KCs, which were then used to establish the epidermal component of organotypic skin cultures. As shown in Figure 1, three different small interfering RNAs (siRNAs) blocked filaggrin mRNA expression by up to 85% and protein expression by more than 95%. The knockdown efficiency was stable over 7 days (Supplementary Figure S1 online). Immunofluorescence labeling of paraffin sections showed strong expression of filaggrin in the granular layers of control organotypic skin samples and absence of filaggrin staining in cultures containing KCs treated with filaggrin-specific siRNAs (Figure 1c).

Histological investigation revealed a complete loss of keratohyalin granules in the filaggrin knockdown organotypic cultures, whereas the morphology of the stratum corneum did not appear to be compromised (Figure 2a and b). Ultrastructural examination confirmed that the number and size of keratohyalin granules was strongly reduced (Figure 2c and e). In addition, electron micrographs showed that the knockdown of filaggrin did not affect the number but the morphology of lamellar bodies. As compared with control samples, lamellar bodies in filaggrin-deficient skin models were smaller (Figure 2d and f) and lacked lamellae of the characteristic shape (Figure 2d, for higher resolution see Supplementary Figure S2 online).

Filaggrin knockdown in organotypic skin cultures affects neither KC differentiation and solubility nor lipid composition

A panel of genes differentially expressed in the various layers of the epidermis was investigated by quantitative real-time PCR (Supplementary Figure S3 online), western blot (Figure 3a), and immunofluorescence analysis (Supplementary Figure S4 online). By comparison with control samples, filaggrin-deficient organotypic skin cultures showed normal mRNA expression levels of loricrin, matriptase-1, and keratins 1, 10, 5, and 14 (Supplementary Figure S3 online). Western blot analysis confirmed that protein expression of involucrin, loricrin, matriptase-1, transglutaminase-1, caspase-14, histidase, and keratins 1, 2, and 10, as well as the small proline-rich molecule SPRR3 was not influenced by filaggrin knockdown in organotypic skin cultures (Figure 3a). A slight increase in the expression of the small proline-rich molecule SPRR2A was detected in the filaggrin-deficient organotypic skin. Immunofluorescence labeling of loricrin, matriptase-1, transglutaminase-1, caspase-14, actin, and keratins 1, 5, 10, and 14 did not reveal differences with regard to the intensity or pattern of labeling between the controls and filaggrin knockdown organotypic skin cultures (Supplementary Figure S4 online). As lipid composition is crucial for epidermal barrier function, we investigated the total lipids extracted from our skin samples. As shown in Figure 3b, we could not detect any significant alterations in the composition of total skin lipids produced by either control or filaggrin knockdown samples using thin layer chromatography. To investigate the solubility of keratins, the organotypic skin models were lysed with buffers containing increasing concentrations of urea, an agent that disrupts intermolecular association. As shown in Figure 4a, comparable amounts of keratins 1 and 10 were found in each urea fraction. In addition, the proteins remaining insoluble after 8 M urea extraction were completely dissolved in 8 M urea buffer containing 2-mercaptoethanol. As shown in Figure 4b, high amounts of keratins were still detectable in this relatively lysis-resistant form in both control and filaggrin-deficient organotypic skin, indicating that filaggrin knockdown did not influence the solubility of keratins 1, 2, and 10 in our model.

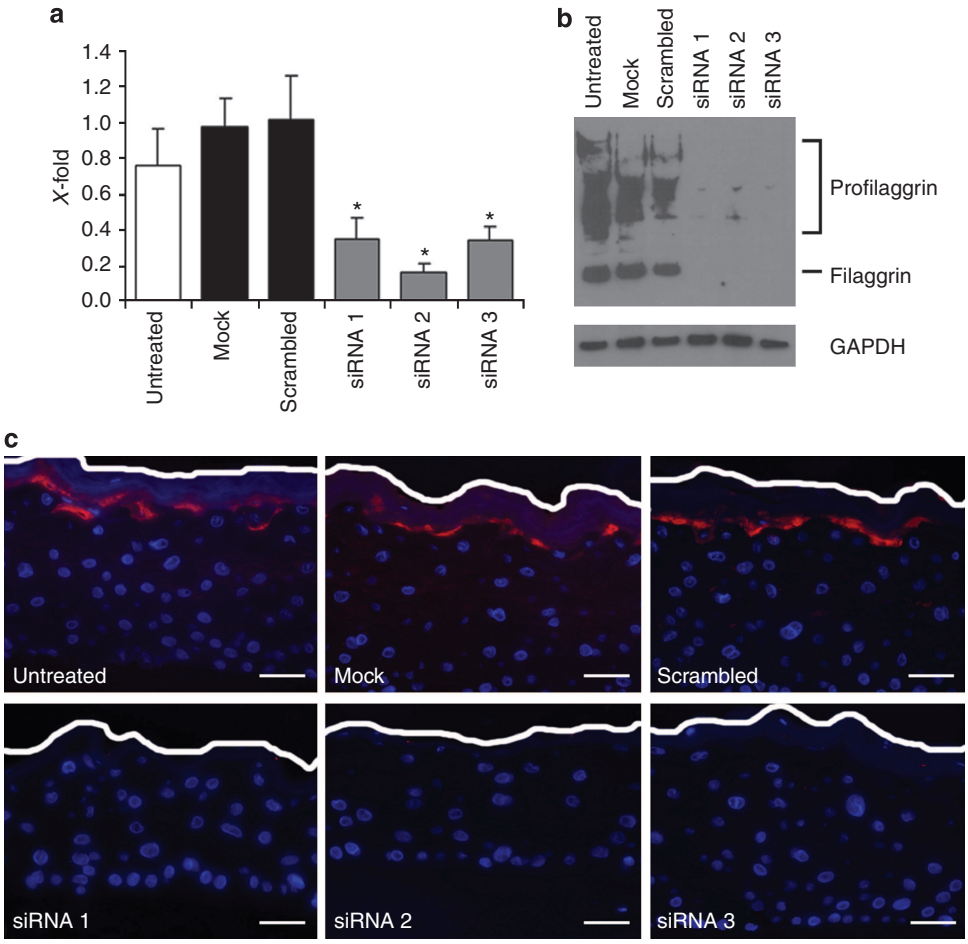

Figure 1. Expression of filaggrin is efficiently blocked by small interfering RNA (siRNA) transfection in organotypic skin cultures. After 7 days in culture, organotypic skin was analyzed by reverse transcription-PCR, western blot analysis, and immunofluorescence staining. (**a**) Filaggrin mRNA production in filaggrin-deficient organotypic skin culture was reduced by more than 60% as compared with control samples. Error bars represent one SD calculated from three replicates for each set of values (*$P<0.001$). (**b**) In contrast to untreated, mock-treated, or scrambled siRNA-treated organotypic skin cultures, filaggrin protein expression was almost completely abolished in organotypic skin cultures transfected with filaggrin-specific siRNAs. Equal loading of protein was controlled by the analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. (**c**) Immunofluorescence staining revealed pronounced filaggrin staining in the granular layers of control organotypic skin cultures, whereas filaggrin expression was virtually absent in organotypic skin cultures from filaggrin siRNA-transfected keratinocytes. One representative experiment of three is shown. Bars = 40 μm.

Lucifer yellow permeability is disturbed in filaggrin-deficient organotypic skin cultures

To investigate Lucifer yellow permeability in our model, we applied the hydrophilic fluorescent dye Lucifer yellow on fully differentiated organotypic skin cultures. As shown in Figure 5, Lucifer yellow penetrated through the stratum corneum down to the basal layer of the filaggrin-deficient skin cultures within 2 hours (Figure 5, lower panel). In contrast, the fluorescent dye did not penetrate through the stratum corneum of the control skin cultures (Figure 5, upper panel).

Filaggrin knockdown decreases the concentration of urocanic acid and increases the UV sensitivity of KCs in the organotypic skin model

Filaggrin contains a high number of histidine residues, which are released by proteolysis of filaggrin and serve as substrates for the formation of urocanic acid (UCA) by histidase. To test the hypothesis that filaggrin is the main source of histidine and indirectly also of UCA in the stratum corneum, we investigated whether the knockdown of filaggrin alters the concentration of UCA in organotypic skin cultures. Indeed, HPLC analysis of organotypic skin lysates revealed a more than 60% decrease in the UCA content in filaggrin knockdown samples as compared with control samples (Figure 6a). As the expression of histidase was not affected by the knockdown of filaggrin (Figure 3a) and UCA is not further metabolized in epidermal cells, the decrease in UCA is likely to be caused by a decrease in histidine.

UCA has been proposed to function as a major UV-absorbing substance of the stratum corneum (Zenisek *et al.*, 1955), suggesting that reduced UCA content may facilitate UV penetration through the stratum corneum. We therefore irradiated normal and filaggrin knockdown skin cultures and compared parameters of UVB-induced cell damage. Compatible with the hypothesis that UCA protects against UVB-induced cell damage, irradiation of filaggrin-deficient skin

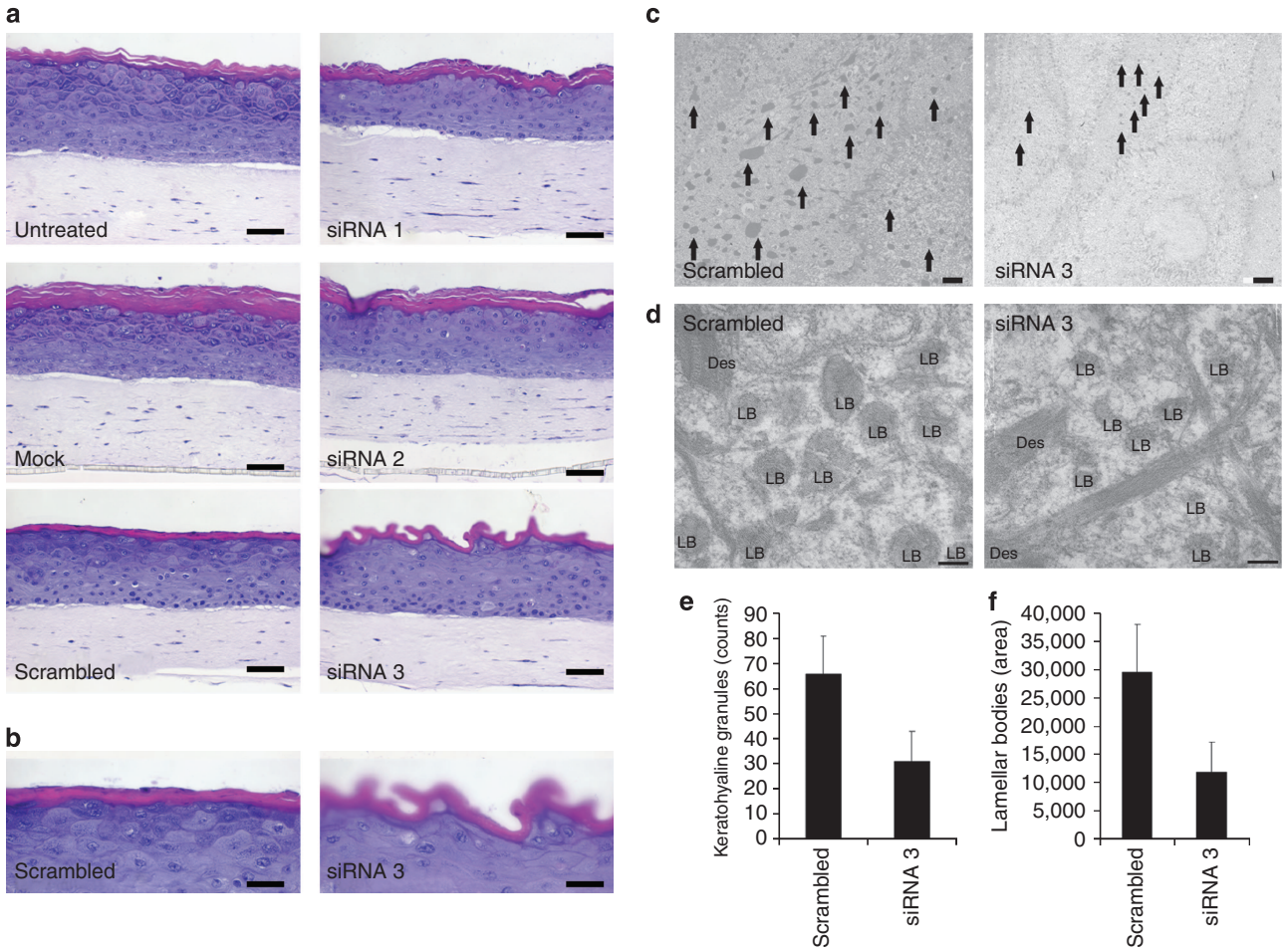

Figure 2. Filaggrin deficiency leads to the loss of keratohyalin granules and impaired lamellar body formation. (**a**, **b**) Hematoxylin and eosin staining of organotypic skin cultures that were untreated, mock treated, or transfected with scrambled small interfering RNA (siRNA), as well as organotypic skin cultures deficient for filaggrin, is shown. Compared to the controls (left panel), keratohyalin granules are completely missing in the filaggrin-deficient organotypic skin cultures (right panel). One representative experiment of three is shown. Bars = 100 μm (**a**), 40 μm (**b**). Transmission electron microscopy reveals fewer and smaller keratohyalin granules ((**c**) arrows) and disturbed lamellar body (LB) formation in the filaggrin-deficient organotypic skin cultures (**d**). Note, most lamellar bodies are smaller and typical lamellae are not visible (**d**). Des, desmosome. Bars = 2 μm (**c**), 100 nm (**d**). (**e**) Number of keratohyaline granules per cell was counted. (**f**) Area of lamellar bodies was measured.

cultures resulted in an increase in both formation of cyclobutane pyrimidine dimers (Figure 6b) and caspase-3 activation (Figure 6c), suggesting increased DNA damage and increased apoptosis.

DISCUSSION

The two most common loss-of-function mutations in the filaggrin gene, R501X and 2282del4, occur in up to 9% of European individuals (Palmer *et al.*, 2006; Smith *et al.*, 2006; McGrath and Uitto, 2008). Considering that several other rare mutations have been discovered recently (Sandilands *et al.*, 2007, 2009), the real mutation carrier frequency is likely to be even higher. Although the association of filaggrin mutations with ichthyosis vulgaris and atopic dermatitis has been well established, the mechanisms leading from genetic defect to clinical manifestations as well as the spectrum of subclinical consequences are still not completely understood. To investigate the function of filaggrin during epidermal differentiation and barrier formation, we used an organotypic skin model (Mildner *et al.*, 2006) and knocked down filaggrin expression by siRNA technology.

Filaggrin knockdown in our model did not influence KC differentiation and stratum corneum development. Except for the small proline-rich molecule, SPRR2A, no differentiation-associated proteins investigated were significantly regulated. SPRR2A expression, however, was slightly increased in the filaggrin-deficient samples. Upregulation of small proline-rich molecules has been demonstrated previously in mice lacking Klf4 (Segre *et al.*, 1999) or loricrin (Koch *et al.*, 2000). In both knockout mice, the development of a functional epidermal barrier is disturbed or at least delayed, suggesting that upregulation of small proline-rich molecules functions as a compensatory mechanism, thereby preventing a more severe phenotype. A similar mechanism might be apparent in our filaggrin-deficient epidermis to overcome the reported diffusion barrier defect.

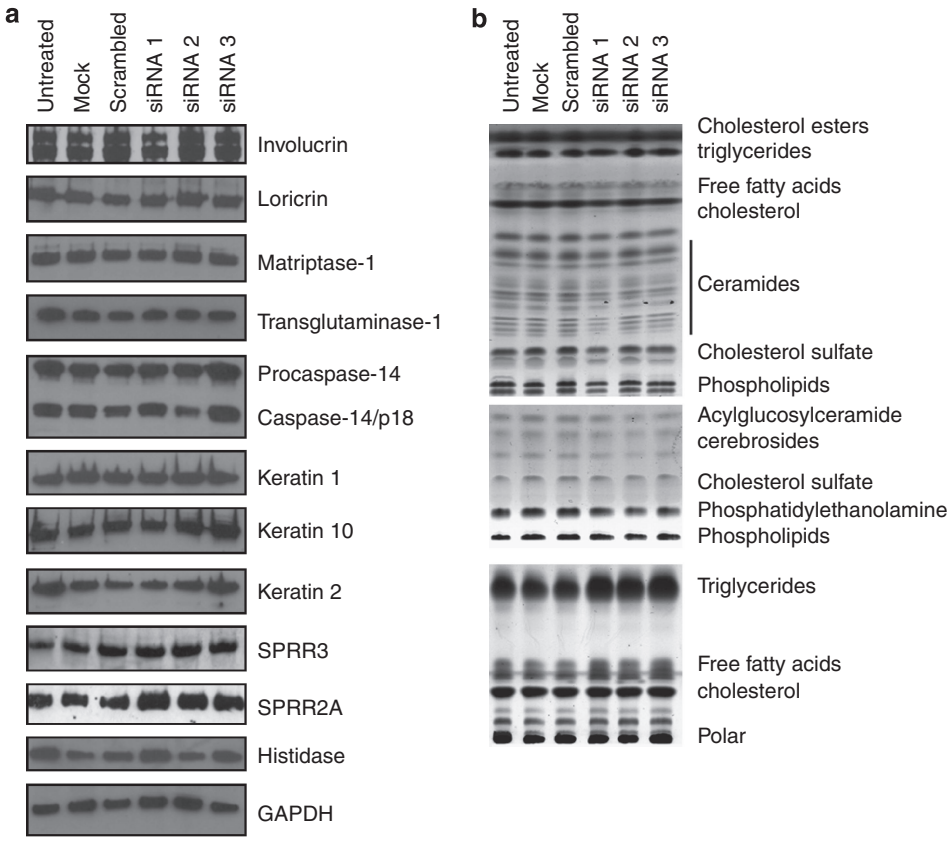

Figure 3. Filaggrin knockdown in organotypic skin cultures does not affect keratinocyte differentiation or the lipid composition. (**a**) Expression of differentiation-associated proteins was analyzed by western blotting. Except of an increased SPRR2A expression all other proteins were not regulated by filaggrin knockdown. Equal loading of protein was controlled by Ponceau staining of the membrane (data not shown), and western blot analysis of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One representative experiment of three is shown. (**b**) Total lipid composition was analyzed by thin layer chromatography. The following solvent systems were used sequentially: upper panel: chloroform/methanol/water 40:10:1 (v/v/v) to 10 cm, chloroform/methanol/acetic acid 190:9:1 (v/v/v) to 16 cm, and hexane/diethylether/acetic acid 70:30:1 (v/v/v) to 20 cm. Middle panel: chloroform/acetone/methanol 76:4:20 (v/v/v) to 2 cm, chloroform/acetone/methanol 80:10:10 (v/v/v) to 15 cm, and chloroform/diethylether/ethylacetate/methanol 72:6:20:2 (v/v/v) to 18 cm. Lower panel: hexane/toluene 1:1 (v/v) to 6 cm and hexane/diethyl ether/acetic acid 80:20:1 (v/v) to 9 cm.

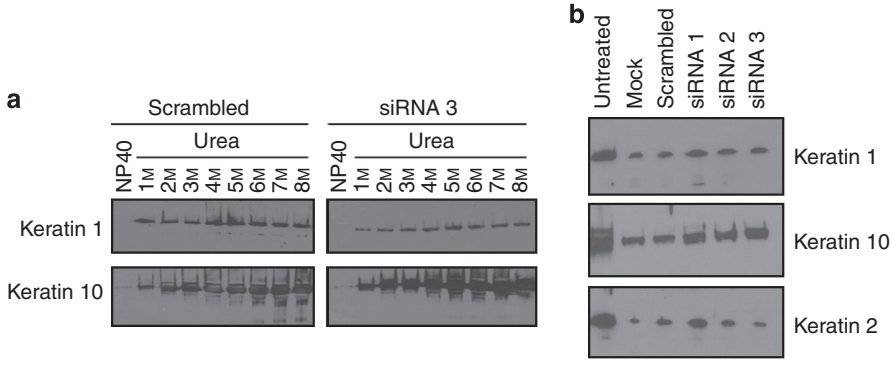

Figure 4. Keratin solubility is not affected by filaggrin knockdown. The solubility of keratins 1, 2, and 10 was analyzed by fractionated lysing of the organotypic skin cultures and western blot analysis. Equal amounts of scrambled small interfering RNA (siRNA) control or filaggrin knockdown organotypic skin cultures were used for the solubility study. Pellets from NP40 buffer lysates were extracted with urea. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) western blot analysis of the corresponding NP40 supernatants confirmed the comparability of the aliquots (data not shown). (**a**) Similar amounts of keratins 1 and 10 were detectable in each urea fraction in both control and siRNA-treated samples. (**b**) The remaining insoluble keratins after 8 M urea extraction were further completely resolved. High amounts of keratins 1, 2, and 10 were detected in both filaggrin knockdown organotypic skin cultures and control samples. One representative experiment of three is shown.

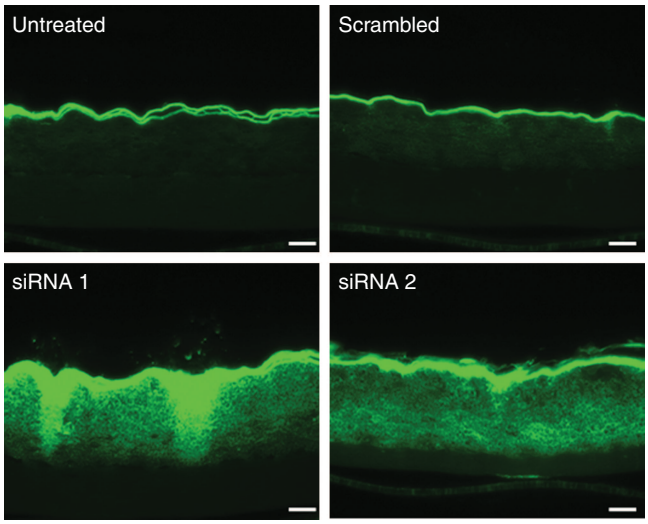

Figure 5. The epidermal diffusion barrier function is disturbed in filaggrin-deficient organotypic skin cultures. After 7 days in culture, 20 µl of the fluorescent dye Lucifer yellow was applied onto the stratum corneum of the organotypic skin cultures and the penetration of the green dye was investigated in thin sections under the fluorescence microscope. Lucifer yellow penetrated through the stratum corneum down to the basal layer of the filaggrin-deficient epidermis but was retained in the stratum corneum of the control samples. Bars = 100 µm.

In accordance with observations in ichthyosis vulgaris, the main morphological alteration in the filaggrin-deficient organotypic skin cultures was the loss of keratohyalin granules. Earlier *in vitro* studies suggested that one of the main functions of this protein is the aggregation of keratin intermediate filaments into tightly aligned keratin bundles (Dale *et al.*, 1978; Steinert *et al.*, 1981), resulting in the name ''filaggrin''. To test for this putative function in our organotypic model, we used previously published protocols (Eichner and Kahn, 1990; Kitahara and Ogawa, 1991, 1997) to assess keratin solubility. We hypothesized that in the absence of filaggrin the aggregation of keratin filaments should be incomplete, resulting in their increased solubility. In contradiction of this hypothesis, our results show that the virtual absence of filaggrin had no impact on the solubility of keratins 1 and 10. Moreover, we emphasize the finding of unaltered solubility of keratin 2, which is expressed only in cells that also express filaggrin. Substitution of filaggrin by other proteins of the S100-fused gene family, such as the recently described filaggrin-2 (Wu *et al.*, 2009), however, is unlikely because these proteins share little sequence similarity to filaggrin (Wu *et al.*, 2009). Consequently, the absence of a keratin aggregation defect in our model indicates that filaggrin is dispensable for keratin aggregation. This conclusion is supported by previous reports showing that keratin filaments seem to be condensed normally in patients with ichthyosis vulgaris (Sybert *et al.*, 1985; Weidenthaler *et al.*, 1993). Although we cannot rule out the possibility that minute amounts of residual filaggrin present after siRNA-mediated knockdown are still sufficient to trigger normal keratin aggregation, our data challenge the key theory that the role of filaggrin is to aggregate keratin filaments. Our results therefore suggest that a comprehensive re-investigation of the interactions between filaggrin and keratins is necessary. In addition, with respect to the proposed function of the filaggrin B-domain in nuclear breakdown (Ishida-Yamamoto *et al.*, 1998; Pearton *et al.*, 2002), our data suggest that filaggrin does not have an essential role for this event.

Recent studies using the *ft* mouse demonstrated that the filaggrin mutation caused disturbed barrier formation and enhanced percutaneous allergen priming (Fallon *et al.*, 2009). These mice have therefore been proposed as a good model not only for ichthyosis vulgaris but also for atopic dermatitis. However, there are some notable differences from human disease: (1) although *ft* mice also show reduced filaggrin expression, the main defect seems to be due to impaired filaggrin processing, (2) the atopic dermatitis-like phenotype was detected only in mice homozygous for the filaggrin mutation (Fallon *et al.*, 2009), whereas in atopic dermatitis patients a heterozygous null filaggrin mutation is sufficient to predispose to both atopic dermatitis and asthma (Henderson *et al.*, 2008). In addition, when studying the barrier function in *ft* mice, it is difficult to rule out confounding factors such as the impact of inflammatory cytokines on barrier function. Our finding that the hydrophilic dye Lucifer yellow is able to penetrate the stratum corneum in filaggrin-deficient organotypic skin suggests that filaggrin deficiency alone is sufficient to impair the function of the epidermal barrier and therefore represents a direct link between these two situations. Scharschmidt *et al.* (2009) have recently demonstrated that in the *ft* mouse the barrier defect is associated with impaired secretion of lamellar bodies as well as altered stratum corneum extracellular membranes, suggesting that this might be the mechanism for impaired barrier function. Indeed, our ultrastructural analysis revealed abnormalities in lamellar body formation. Most of the lamellar bodies were smaller and lacked their typical lamellae. However, our analysis of lipids did not show differences between normal and filaggrin-deficient epidermis. This indicates that the barrier defect of filaggrin-deficient skin models is not caused by an abnormality in lipid composition. Notably, the epidermis of human carriers of filaggrin mutations was reported to have a lipid composition similar to that of individuals with normal filaggrin (Jungersted *et al.*, 2010). Nevertheless, the absence of filaggrin may cause abnormalities in the metabolism and localization of lipids that become apparent only upon sophisticated analysis. Further investigations are necessary to identify the mechanism by which filaggrin deficiency leads to impaired lamellar body formation and to determine the molecular mechanism of the defect in the dye diffusion barrier.

Filaggrin is enzymatically degraded within the stratum corneum, giving rise to free amino acids, some of which are processed further. One of these products is UCA, derived from histidine by the action of stratum corneum histidase (Furuta *et al.*, 1996), and thought to act as an important UV absorbent within the stratum corneum (Zenisek *et al.*, 1955). Our finding that knockdown of filaggrin in our skin model leads to a strong decrease of UCA supports the concept that

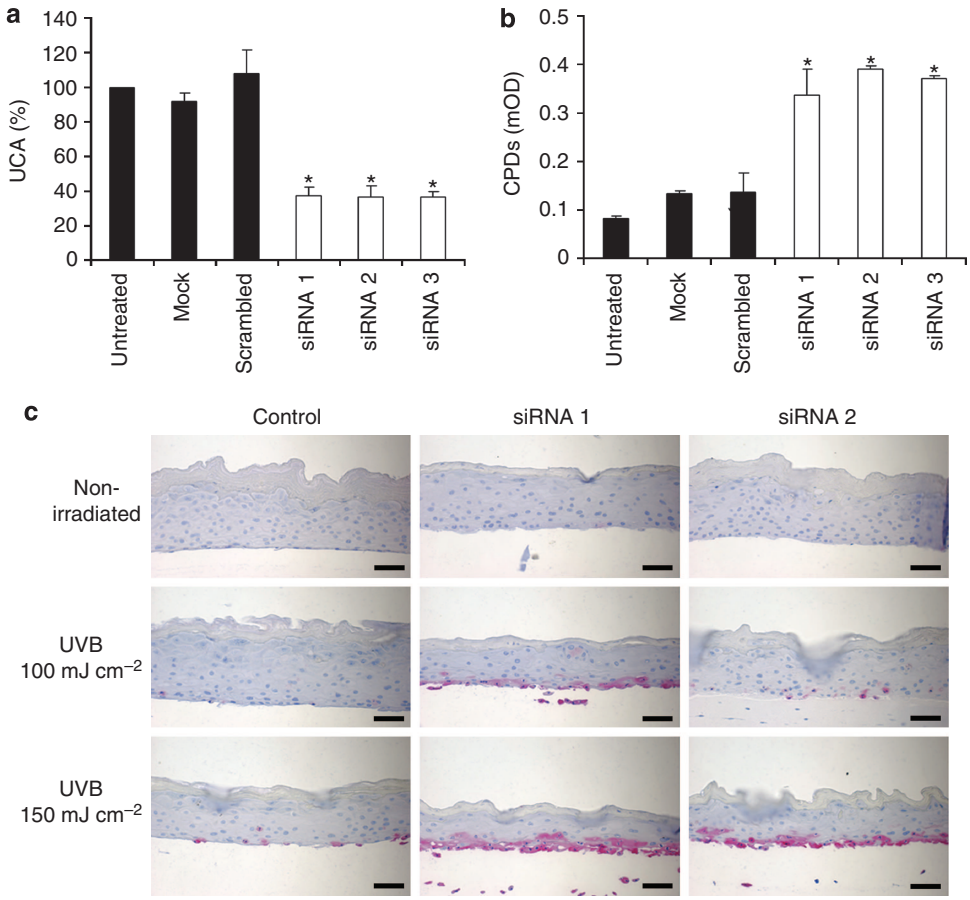

Figure 6. Decreased urocanic acid (UCA) production and increased UV sensitivity in filaggrin-deficient organotypic skin cultures. (**a**) After 7 days in culture, organotypic skin were lysed in 1 M KOH and subjected to HPLC. Filaggrin deficiency resulted in a 60% reduction of the UCA content in the filaggrin knockdown samples. Error bars represent one SD calculated from three experimental replicates for each set of values each carried out in duplicate (*$P<0.001$). (**b**) Exposure to UVB (20 mJ cm^{-2}) led to an increased cyclobutane pyrimidine dimer (CPD) formation in filaggrin knockdown samples after exposure to UVB. Error bars represent one SD calculated from two experimental replicates for each set of values each carried out in triplicate (*$P<0.01$). (**c**) Immunohistochemistry for active caspase-3 revealed an enhanced activation of caspase-3, a marker of apoptosis, in filaggrin-deficient organotypic skin cultures after exposure to UVB (100 and 150 mJ cm^{-2}). Bars = 100 μm.

filaggrin is the main source of UCA in the stratum corneum. In line with the hypothesis that UCA contributes to UV protection, filaggrin knockdown epidermis showed increased sensitivity to UVB-induced KC damage. Although it remains to be investigated whether factors other than UCA also contribute to this phenomenon, our data establish a (most likely indirect) role of filaggrin in photoprotection of the epidermis. It is noteworthy at this point that topical application of UCA strongly decreased photosensitivity both in the presence and absence of normal concentrations of endogenous UCA (our unpublished data). Our results predict that the defective stratum corneum in filaggrin-deficient skin allows more UVB to reach viable KC than would normal stratum corneum, thereby inducing higher levels of DNA damage. A similar phenomenon has been recently described in caspase-14 knockout animals (Denecker *et al.*, 2007). Interestingly, these mice show impaired filaggrin processing leading to impaired barrier function and increased UV sensitivity (Denecker *et al.*, 2007). In contrast to our *in vitro* data, only a few reports show that patients with atopic dermatitis or ichthyosis vulgaris may have increased UVB photosensitivity (Tajima *et al.*, 1998; Murphy, 2001; ten Berge *et al.*, 2009). It should be noted, however, that none of these studies correlated the presence of filaggrin mutations with the outcome of the photosensitivity tests. In addition, compensatory mechanisms *in vivo* might be able to substitute for diminished protection against UVB due to the reduction of UCA. For example, in ichthyosis vulgaris (Tomita *et al.*, 2005) the stratum corneum is thicker than that of normal skin. Thickening of the stratum corneum is regularly also observed in chronic lesions of atopic eczema and in clinically normal skin of atopic dermatitis patients with filaggrin mutations (Nemoto-Hasebe *et al.*, 2008). In both diseases, the increase of stratum corneum thickness may compensate for the diminished UV protection by the reduction/absence of UCA. To address the role of filaggrin and its derivatives in UV protection, specific studies on the photosensitivity of individuals with functional mutations of the filaggrin gene, taking into account the stratum corneum changes, will be required.

In summary, this study demonstrates that filaggrin deficiency by itself is sufficient to impair epidermal barrier

formation, and that absence of filaggrin results in enhanced UV sensitivity, most likely due to the reduction of epidermal UCA.

MATERIALS AND METHODS

Cell culture and siRNA transfection

Normal human dermal fibroblasts (Lonza, Basel, Switzerland) and normal human epidermal KCs (Lonza) were cultured as described previously (Mildner *et al.*, 2006). Three Stealth siRNAs specific for filaggrin and a scrambled control were obtained from Invitrogen (Carlsbad, CA). The siRNA sequences are listed in Supplementary Table S1 online. For siRNA transfection, see Supplementary materials and methods online.

Preparation of organotypic knockdown skin cultures

In vitro organotypic skin cultures were generated as previously described (Mildner *et al.*, 2006). For details see Supplementary materials and methods online.

RNA isolation and reverse transcription-PCR

Organotypic epidermis was lysed by TRIzol reagent (Invitrogen) and RNA was extracted following the manufacturer's instructions. Reverse transcription-PCR was performed using iScript cDNA Synthesis Kit (BIO-RAD Laboratories, Hercules, CA) according to the manufacturer's instructions.

Quantitative real-time PCR

To measure filaggrin knockdown efficiency on the mRNA level and to investigate the mRNA expression of other differentiation-associated genes, quantitative real-time PCR was performed with LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. The primers used are listed in Supplementary Table S2 online. The relative expression of the target genes was calculated by comparing with the housekeeping gene β-2-microglobulin using a formula described by Pfaffl (2001).

Antibodies

All antibodies used are listed in Supplementary Table S3 online.

SDS–PAGE and western blot

Knockdown efficiency of filaggrin on the protein level and the protein expression levels of other differentiation-associated genes were analyzed by SDS–PAGE and western blot as described previously (Mildner *et al.*, 2002). For details see Supplementary materials and methods online.

Keratin solubility

Keratin solubility was determined according to previously published protocols (Eichner and Kahn, 1990; Kitahara and Ogawa, 1991, 1997). For details see Supplementary materials and methods online.

Lipid extraction and thin layer chromatography

Organotypic skin was homogenized and total lipids extracted according to a modification of the Folch method (Folch *et al.*, 1957) (chloroform/methanol/formic acid 2:1:0.5 v:v:v) and taken up in chloroform. Total lipid extracts of organotypic skin cultures were separated by one-dimensional thin layer chromatography as described previously by Ponec *et al.* (1988), Horsley *et al.* (2006), and Pappinen *et al.* (2008). Thin layer chromatography separation is detailed in the Supplementary materials and methods online.

Transmission electron microscopy

Organotypic skin cultures for transmission electron microscopy were fixed in Karnovsky's fixative according to the standard procedures and embedded in epon resin. Ultrathin sections (0.07 μm) were cut, stained with uranyl acetate and lead citrate, and observed with a JEOUL 1010 transmission electron microscope (Jeoul, Peabody, MA) at 60 kV and photographed with the camera incorporated in the microscope.

Analysis of Lucifer yellow permeability

At day 7 after initiation of the organotypic skin culture, 20 μl Lucifer yellow (Sigma, 1 mM, Vienna, Austria) were added onto the stratum corneum of the organotypic skin samples and incubated at 37 °C for 2 hours. The artificial skin samples were fixed in 3.7% formaldehyde and embedded in paraffin. Sections (5 μm) were inspected under the fluorescence microscope.

UV irradiation

Organotypic skin models were cultured for 7 days and exposed to UVB (280–320 nm). For irradiation details see Supplementary materials and methods online.

Analysis of cyclobutane pyrimidine dimers

Cyclobutane pyrimidine dimers were determined quantitatively by ELISA according to a protocol recommended by MBL International Corporation (Woburn, MA) with modifications. For details see Supplementary materials and methods online.

Analytical determination of the UCA content by HPLC

Organotypic skin cultures were lysed in 0.1 M KOH and further analyzed by HPLC as described previously (Kezic, 2009).

Statistical analysis

The data were analyzed using Student's *t*-test with $P = 0.05$ being defined as the level of significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Ms H. Rossiter for critical reading of the paper. This project was supported by the European Union COST initiative on skin barrier in atopic dermatitis.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* 6:328–40

Dale BA, Holbrook KA, Steinert PM (1978) Assembly of stratum corneum basic protein and keratin filaments in macrofibrils. *Nature* 276:729–31

Dale BA, Presland RB, Lewis SP *et al.* (1997) Transient expression of epidermal filaggrin in cultured cells causes collapse of intermediate filament networks with alteration of cell shape and nuclear integrity. *J Invest Dermatol* 108:179–87

Denecker G, Hoste E, Gilbert B *et al.* (2007) Caspase-14 protects against epidermal UVB photodamage and water loss. *Nat Cell Biol* 9:666–74

Denecker G, Ovaere P, Vandenabeele P *et al.* (2008) Caspase-14 reveals its secrets. *J Cell Biol* 180:451–8

Eichner R, Kahn M (1990) Differential extraction of keratin subunits and filaments from normal human epidermis. *J Cell Biol* 110:1149–68

Fallon PG, Sasaki T, Sandilands A *et al.* (2009) A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. *Nat Genet* 41:602–8

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497–509

Furuta T, Okamiya K, Shibasaki H *et al.* (1996) Pharmacokinetics of stable isotopically labeled L-histidine in humans and the assessment of *in vivo* histidine ammonia lyase activities. *Drug Metab Dispos* 24:49–54

Gan SQ, McBride OW, Idler WW *et al.* (1990) Organization, structure, and polymorphisms of the human profilaggrin gene. *Biochemistry* 29:9432–40

Harding CR, Scott IR (1983) Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. *J Mol Biol* 170:651–73

Henderson J, Northstone K, Lee SP *et al.* (2008) The burden of disease associated with filaggrin mutations: a population-based, longitudinal birth cohort study. *J Allergy Clin Immunol* 121:872–7

Horsley V, O'Carroll D, Tooze R *et al.* (2006) Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland. *Cell* 126:597–609

Ishida-Yamamoto A, Takahashi H, Presland RB *et al.* (1998) Translocation of profilaggrin N-terminal domain into keratinocyte nuclei with fragmented DNA in normal human skin and loricrin keratoderma. *Lab Invest* 78:1245–53

Jungersted JM, Scheer H, Mempel M *et al.* (2010) Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy*; e-pub ahead of print 4 February 2010

Kezic S, Kammeyer A, Calkoen F *et al.* (2009) Natural moisturizing factor components in the stratum corneum as biomarkers of filaggrin genotype: evaluation of minimally invasive methods. *Br J Dermatol* 161:1098–104

Kitahara T, Ogawa H (1991) The extraction and characterization of human nail keratin. *J Dermatol Sci* 2:402–6

Kitahara T, Ogawa H (1997) Cellular features of differentiation in the nail. *Microsc Res Tech* 38:436–42

Koch PJ, de Viragh PA, Scharer E *et al.* (2000) Lessons from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein. *J Cell Biol* 151:389–400

McGrath JA, Uitto J (2008) The filaggrin story: novel insights into skin-barrier function and disease. *Trends Mol Med* 14:20–7

Mehrel T, Hohl D, Rothnagel JA *et al.* (1990) Identification of a major keratinocyte cell envelope protein, loricrin. *Cell* 61:1103–12

Mildner M, Ballaun C, Stichenwirth M *et al.* (2006) Gene silencing in a human organotypic skin model. *Biochem Biophys Res Commun* 348:76–82

Mildner M, Eckhart L, Lengauer B *et al.* (2002) Hepatocyte growth factor/scatter factor inhibits UVB-induced apoptosis of human keratinocytes but not of keratinocyte-derived cell lines via the phosphatidylinositol 3-kinase/AKT pathway. *J Biol Chem* 277:14146–52

Murphy GM (2001) Diseases associated with photosensitivity. *J Photochem Photobiol B* 64:93–8

Nemoto-Hasebe I, Akiyama M, Nomura T *et al.* (2008) Clinical severity correlates with impaired barrier in filaggrin-related eczema. *J Invest Dermatol* 129:682–9

Palmer CN, Irvine AD, Terron-Kwiatkowski A *et al.* (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 38:441–6

Pappinen S, Hermansson M, Kuntsche J *et al.* (2008) Comparison of rat epidermal keratinocyte organotypic culture (ROC) with intact human skin: lipid composition and thermal phase behavior of the stratum corneum. *Biochim Biophys Acta* 1778:824–34

Pearton DJ, Dale BA, Presland RB (2002) Functional analysis of the profilaggrin N-terminal peptide: identification of domains that regulate nuclear and cytoplasmic distribution. *J Invest Dermatol* 119:661–9

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45

Ponec M, Weerheim A, Kempenaar J *et al.* (1988) Lipid composition of cultured human keratinocytes in relation to their differentiation. *J Lipid Res* 29:949–61

Presland RB, Boggess D, Lewis SP *et al.* (2000) Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. *J Invest Dermatol* 115:1072–81

Presland RB, Dale BA (2000) Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Med* 11:383–408

Presland RB, Haydock PV, Fleckman P *et al.* (1992) Characterization of the human epidermal profilaggrin gene. Genomic organization and identification of an S-100-like calcium binding domain at the amino terminus. *J Biol Chem* 267:23772–81

Presland RB, Kimball JR, Kautsky MB *et al.* (1997) Evidence for specific proteolytic cleavage of the N-terminal domain of human profilaggrin during epidermal differentiation. *J Invest Dermatol* 108:170–8

Presland RB, Kuechle MK, Lewis SP *et al.* (2001) Regulated expression of human filaggrin in keratinocytes results in cytoskeletal disruption, loss of cell-cell adhesion, and cell cycle arrest. *Exp Cell Res* 270:199–213

Sandilands A, Sutherland C, Irvine AD *et al.* (2009) Filaggrin in the frontline: role in skin barrier function and disease. *J Cell Sci* 122:1285–94

Sandilands A, Terron-Kwiatkowski A, Hull PR *et al.* (2007) Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 39:650–4

Scharschmidt TC, Man MQ, Hatano Y *et al.* (2009) Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. *J Allergy Clin Immunol* 124:496–506

Scott IR, Harding CR (1986) Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. *Dev Biol* 115:84–92

Scott IR, Harding CR, Barrett JG (1982) Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. *Biochim Biophys Acta* 719:110–7

Segre JA, Bauer C, Fuchs E (1999) Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet* 22:356–60

Smith FJ, Irvine AD, Terron-Kwiatkowski A *et al.* (2006) Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat Genet* 38:337–42

Steinert PM, Cantieri JS, Teller DC *et al.* (1981) Characterization of a class of cationic proteins that specifically interact with intermediate filaments. *Proc Natl Acad Sci USA* 78:4097–101

Sybert VP, Dale BA, Holbrook KA (1985) Ichthyosis vulgaris: identification of a defect in synthesis of filaggrin correlated with an absence of keratohyaline granules. *J Invest Dermatol* 84:191–4

Tajima T, Ibe M, Matsushita T *et al.* (1998) A variety of skin responses to ultraviolet irradiation in patients with atopic dermatitis. *J Dermatol Sci* 17:101–7

ten Berge O, van Weelden H, Bruijnzeel-Koomen CA *et al.* (2009) Throwing a light on photosensitivity in atopic dermatitis: a retrospective study. *Am J Clin Dermatol* 10:119–23

Tomita Y, Akiyama M, Shimizu H (2005) Stratum corneum hydration and flexibility are useful parameters to indicate clinical severity of congenital ichthyosis. *Exp Dermatol* 14:619–24

Watt FM (1983) Involucrin and other markers of keratinocyte terminal differentiation. *J Invest Dermatol* 81:100s–3s

Weidenthaler B, Hausser I, Anton-Lamprecht I (1993) Is filaggrin really a filament-aggregating protein *in vivo*? *Arch Dermatol Res* 285:111–20

Wu Z, Hansmann B, Meyer-Hoffert U *et al.* (2009) Molecular identification and expression analysis of filaggrin-2, a member of the S100 fused-type protein family. *PLoS One* 4:e5227

Zenisek A, Kral JA, Hais IM (1955) Sun-screening effect of urocanic acid. *Biochim Biophys Acta* 18:589–91